Charles University, Prague
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The study of molecular and biochemical bases of cytochrome *c* oxidase deficiency

PhD Thesis

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INTRODUCTION

Energy generation

The adequate energy production is a crucial ability and necessity for any living cell. One of the elementary metabolic processes involved in energy generation is glycolysis. With variations, it occurs in many types of cells in nearly all organisms. Via glycolysis is energy in the form of energy-rich molecules (ATP) and electron carriers ($NADH+H^+$) generated by oxidation of glucose to pyruvate (Champe, 2005).

In anaerobic conditions, pyruvate is converted by fermentation to waste products as lactic acid or ethanol and carbon dioxide. The final pyruvate conversion is important for the proper cell function, while it is the only process to remove hydrogens and electrons from the $NADH+H^+$ and regenerate NAD^+ (nicotinamide adenine dinucleotide) for another glycolysis to proceed. Lactate oxidation is especially important for human cells in the situations when glycolysis is the only source of ATP – during the fetal life period or later in severely-hypoxic conditions (Alberts, 1994; Hay, 2006; Champe, 2005; Makinde et al., 1998).

In aerobic conditions in most eukaryotic cells, pyruvate is transported to the mitochondria, where it is converted by the pyruvate dehydrogenase complex to acetyl coenzyme A (acetyl-CoA) (Alberts, 1994; Champe, 2005). As a substrate, acetyl-CoA enters another series of enzyme-catalyzed chemical reactions - the citric acid cycle, also known as the Krebs cycle or the tricarboxylic acid cycle (TCA). Krebs cycle substrates do not come only from glycolysis, but also from beta-oxidation of fat acids and proteolysis (Champe, 2005). In all living cells, that use oxygen as a part of cellular respiration, Krebs cycle plays highly important role. For each acetyl group that enters the citric acid cycle, three molecules of $NADH+H^+$ are produced. The Krebs cycle step of succinate oxidation to fumarate, catalyzed by succinate dehydrogenase, use flavin adenine nucleotide - FAD^+ as the acceptor of electrons by forming $FADH_2$ (Champe, 2005). All produced $NADH+H^+$ and $FADH_2$ donate electrons to the mitochondrial respiratory chain and ATP synthase processes called oxidative phosphorylation (OXPHOS) system (Kadenbach et al., 2000). Such an aerobic oxidation of carbohydrates, proteins, and fats connected with the efficient oxidative phosphorylation system, is markedly more efficient in generation of ATP than just glycolysis (Fontanesi et al., 2006).

Mitochondria

A mitochondrion is an organelle found in most of eukaryotic cells (Henze and Martin, 2003). Mitochondria are known as “powerhouses” of eukaryotic cells, because they generate most of the cell’s supply of the chemical energy source – of the ATP (Saraste, 1999). In addition to the metabolisms of proteins, fats, amino acids, porphyrins and many other substances, mitochondria are involved also in processes, such as signaling, cellular differentiation, cell death, controlling of the cell cycle and cell growth as well as in the aging process (McBride et al., 2006; Szibor and Holtz, 2003).

Functional and phylogenetic studies of mitochondria clearly demonstrate their eubacterial origin (Chihade et al., 2000). Mitochondria are believed to be primeval interlopers, primitive bacteria that fostered an endosymbiotic relationship with early eukaryotic cells and eventually became integral to the normal function of those cells (McFarland et al., 2007). Mitochondria have themselves evolved and developed into highly specialized organelles with a key role in eukaryotic cell metabolism. Their primary function of energy conversion is indispensable to every eukaryotic cell. However, the relationship is reciprocal and mitochondria are critically reliant on the import of molecules from the cytosol for a variety of specialized purposes (McFarland et al., 2007). Mitochondria in largely postmitotic cells (e.g. cardiomyocytes, neurons or skeletal muscle cells) have a limited life span of a few weeks (Szibor and Holtz, 2003).

Mitochondria range from 1 to 10 micrometers in size (Vogel et al., 2006). As a cellular organelle, it consists of a continuous reticulum that makes up nearly 10% of the cell volume in respiring yeast cells (Yaffe, 1999). A double membrane, which encloses the organelle, creates two internal compartments (Frey and Mannella, 2000). The space between the two membranes is called the intermembrane space and is interrupted by junction points in which the inner membrane and outer membrane are in contact. The volume enclosed within the inner membrane is known as the matrix compartment. In cells with high respiration rates, the inner membrane is convoluted; folding into tubular structures designated cristae (Cobine et al., 2006b). The cristae tubules are 30–40 nm in diameter (Frey and Mannella, 2000). The

mitochondrial reticulum is highly dynamic, constantly changing size and shape through fission and fusion events (Hermann and Shaw, 1998).

The outer membrane is a relatively simple and presents barrier only for macromolecules. Therefore, it contains pore-forming proteins, which render it permeable to molecules up to few thousand Dalton (Vogel et al., 2006). Ions, nutrient molecules, ATP, ADP, etc. can pass easily through the outer membrane.

The inner membrane is freely permeable only to oxygen, carbon dioxide, and water. For other components it presents highly efficient barrier and they must enter the matrix via special transporting proteins and systems. It is one of the most protein-rich lipid bilayers in biological system containing various multisubunit protein complexes, which also perform several cell fundamental processes (Vogel et al., 2006). The most abundant proteins are complexes of the OXPHOS. Over 80 different polypeptides interact on the inner mitochondrial membrane to form the respiratory chain (Chinnery and Turnbull, 2001). The rest of the inner membrane proteins are a specialized transporting proteins.

The mitochondrial matrix houses plenty of enzymes involved in important processes of the metabolism of carbohydrates and fats, such as enzymes of the Krebs cycle and the β-oxidation.

Fig. 1 Mitochondria
a) scheme (inner membrane (IM), intermembrane space (IMS), outer membrane (OM) and matrix),(www.protein-ms.de/RES/Mito/mitoown.JPG); b) transmission electron microscopy in cultured human skin fibroblast, 40000x, (by the courtesy of Dr. Sládková); c) mitochondrial reticulum stained by MitoTracker Red, (by the courtesy of Dr. Sládková).

The biochemistry of mitochondria has been the subject of intense investigation over the past 50 years (McBride et al., 2006; Yaffe, 1999). Within these organelles, not only sugars and long chain fatty acids are broken down, but also ADP is recycled

back into ATP, steroids and lipids are synthesized along with numerous other reactions that are essential for human life (McBride et al., 2006).

A unique feature of mitochondria is the presence of a separate genome, mitochondrial DNA (mtDNA), which is distinct from that of the nucleus (Lee and Sokol, 2007). mtDNA is a circular double stranded molecule of 16 569 base pairs placed in the mitochondrial matrix. Genome encodes two ribosomal RNAs, 22 transfer RNAs for protein synthesis and the 13 polypeptides (Szibor and Holtz, 2003). mtDNA does not have introns. Each mitochondrion contains several copies of mtDNA and every cell has many mitochondria, so there are hundreds of copies of mtDNA in one cell (polyplasmy). A human hepatocyte contains approximately 1000 copies of mtDNA (Lee and Sokol, 2007). However, mtDNA depends on nuclear genes for its enzymes of replication, transcription, translation and repair, including DNA polymerase γ, thymidine kinase 2, and deoxyguanosine kinase (Capps et al., 2003; DiMauro and Schon, 2003). Mitochondria rely upon the nucleus for all the other proteins involved in the many metabolic pathways that occur in this organelle (Leonard and Schapira, 2000).

In mammals all functional mtDNAs are inherited from the mother so it presents maternal inheritance. Human mtDNA has a mutation rate 10-20 times higher that of nuclear DNA, probably due to failure of proof-reading by mtDNA polymerases (Finsterer, 2006). Normally all of an individual's mtDNA is identical (homoplasmy). Occasionally, a sequence variation creates a dual population of wild-type and mutant mtDNA (heteroplasmy). It implies potentially harmful mutant mtDNA (Leonard and Schapira, 2000). Heteroplasmy is enhanced by the stochastic distribution of the mtDNA to daughter cells, resulting in changing mutation loads in consecutive generations and increasing the phenotypic variation of mitotic segregation. Because of mitotic segregation and polyploidy, phenotypic expression is dependent on a threshold effect. If the load of mutant mtDNA copies exceeds a certain amount, the effect of a mutation can no longer be compensated by the wild-type. mtDNA mutations include point mutations and small or large-scale rearrangements (deletions and duplications) (Zeviani and Di Donato, 2004). Since there are no introns, there are no splice-side mutations. Point mutations are commonly maternally inherited whilst deletions and duplications are most often sporadic. Disease expression is determined by the percentage of mutant mtDNA in a given cell or tissue, which may differ substantially among tissues (Lee and Sokol, 2007). The phenotypic variability is

also dependent on the pathogenicity of a mutation, the affected gene, its tissue distribution, and the dependency of an organ on the mitochondrial energy supply. Organs, such as the eyes, ears, central and peripheral nervous systems, heart, endocrine system, kidney, guts, and liver are commonly affected (Finsterer, 2006).

Frequent fusion and fission within a dynamic network may be an efficient means of intermitochondrial DNA (mtDNA) complementation through exchange of genomes between fusing mitochondria (Nakada et al., 2001). Introduction of mitochondria bearing mtDNA mutations that inactivate cytochrome *c* oxidase (COX) into cells with wild-type mitochondria, resulted in dynamic mixing of matrix and membrane proteins, as well as mtDNA transfer, between normal- and COX-activity-deficient organelles so that all mitochondria within the cell displayed comparable, high COX activity (Nakada et al., 2001). mtDNA is prone to mutations that accumulate in aging individuals (Nakada et al., 2001). Genetic complementation, likely achievable due to the dynamic network-like behavior, may represent a mechanism of repairing mtDNA mutations, and consequently maintain properly functioning oxidative complexes within the cell (Karbowski and Youle, 2003).

Respiratory chain, oxidative phosphorylation and ATP synthesis

The respiratory chain and OXPHOS system contains 83 polypeptides. 70 are encoded by nuclear genes; the other 13 are encoded by mtDNA (Leonard and Schapira, 2000). All together, they form of five multimeric protein complexes:

Complex I – NADH:ubiquinone oxidoreductase (containing approximately 46 subunits, 7 of them encoded by mtDNA)

Complex II – succinate:ubiquinone oxidoreductase (4 subunits), which catalyze also the Krebs cycle step of succinate oxidation to fumarate

Complex III – decylubiquinol-cytochrome c oxidoreductase (11 subunits, one of them encoded by mtDNA)

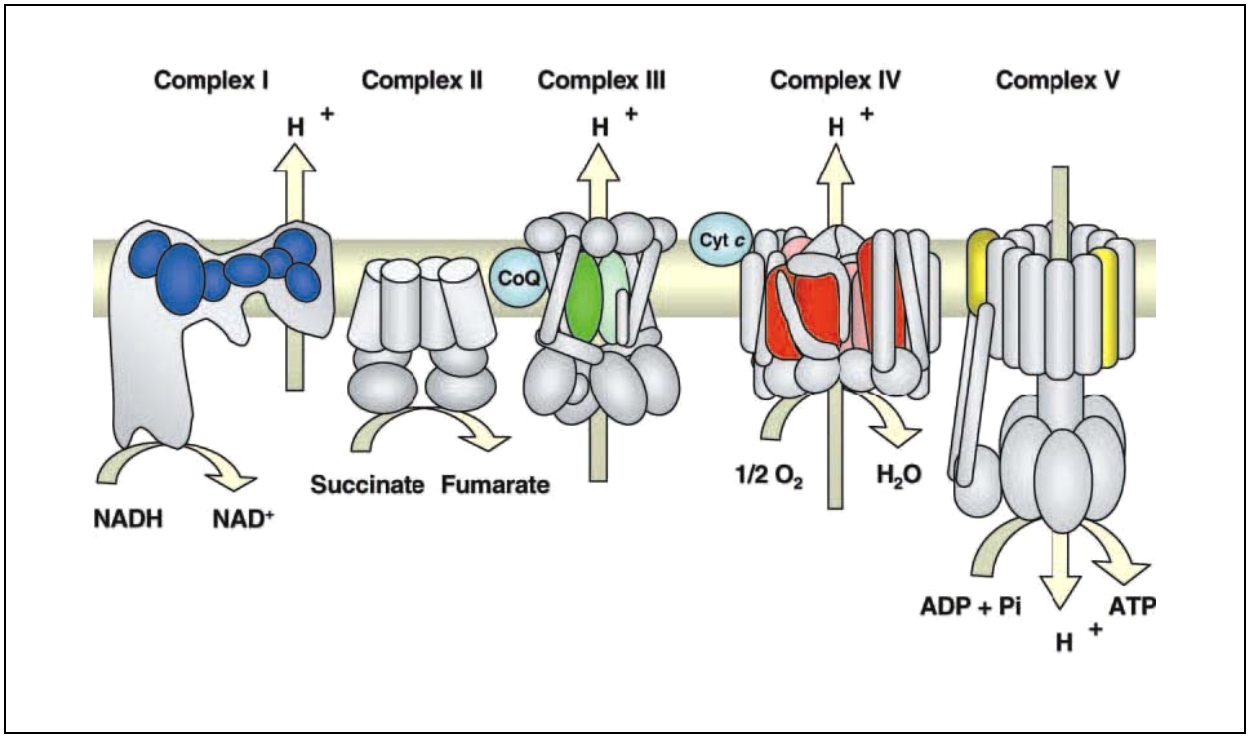
Complex IV – cytochrome c oxidase (13 subunits, 3 of them encoded by mtDNA)

ATP synthetase - complex V (approximately 16 subunits, 2 of them encoded by mtDNA), which is in fact an independent protein complex but its function is closely coupled to respiratory chain. Therefore some authors name it complex V and some authors strictly exclude this complex from respiratory chain complexes terminology.

Respiratory chain also requires two small electron carriers – ubiquinone (coenzyme Q_{10}) and cytochrome c that shuttles electrons between large, relatively immobile macromolecular complexes imbedded in the membrane (Saraste, 1999).

Each of these complexes incorporates multiple electron carriers. Complexes I, II, and III contain several Fe - S centers, while complexes III and IV include cytochromes (Wallace, 2001).

Fig. 2 Creative drawing of the respiratory chain complexes. (Zeviani and Di Donato, 2004) Mitochondrially encoded subunits, embedded in the midst of nuclear-encoded subunits, are shown in different colors: complex I subunits = blue; complex III subunit = green; complex IV subunits = red; complex V subunits = yellow. Pi = inorganic phosphate; Cyt c = cytochrome c; CoQ = coenzyme Q.



The mitochondria oxidize hydrogen to generate water and ATP. Reducing equivalents produced by the Krebs cycle and by β-oxidation are transferred to NAD^+, generating $NADH+H^+$, or to flavin adenine nucleotide (FAD^+), generating $FADH_2$. $NADH+H^+$ donates electrons to respiratory chain complex I, whereas $FADH_2$ cedes electrons, either from succinate (in the Krebs cycle) to complex II or from reduced electron transfer protein (at the end of β-oxidation) directly to ubiquinone (coenzyme Q_{10}). Ubiquinol transfers its electrons to complex III, where the electrons are picked up by cytochrome c. Cytochrome c brings the electrons to complex IV and finally the electrons are used together with ½ O_2 to give rise of H_2O molecule. The energy released by this process is used to pump protons (by complexes I, III and IV) from

the matrix to the intermembrane space, creating the transmembrane electrochemical gradient. The potential energy stored in the electrochemical gradient is used to condense ADP and inorganic phosphate to make ATP via complex V (ATP synthase), driven by the movement of protons back through a complex V proton channel (DiMauro et al., 2006; Fontanesi et al., 2006; Chinnery and DiMauro, 2005; Lee and Sokol, 2007; van den Heuvel and Smeitink, 2001; Wallace, 2001). Some of the physical energy of the proton gradient is used to generate heat. Produced ATP presents a readily utilizable and essential energy source for the eukaryotic cell (McFarland et al., 2007).

The amount of energy released by oxidative phosphorylation is high, compared with the amount produced by anaerobic fermentation. Glycolysis produces only 2 ATP molecules, but almost 30 ATPs are produced by the oxidative phosphorylation of the 10 NADH and 2 succinate molecules made by converting one molecule of glucose to carbon dioxide and water (Rich, 2003). This ATP yield is the theoretical maximum value; in practice, some protons leak across the membrane, lowering the yield of ATP (Porter, 2001).

The exclusive niche that mitochondria occupy in the metabolism of the eukaryotic cell is central to their role in human disease. The cells dependence on ATP derived from OXPHOS makes it extremely vulnerable to mitochondrial malfunction and even small reductions in the efficiency of ATP production in some tissues may have functional impact to the affected tissue.

The OXPHOS system, which comprises the electron transport chain and ATP synthase, is the only cellular pathway under dual genetic control (DiMauro et al., 2006). This fact can theoretically give rise to very variable symptoms, in any organ or tissue, at any age with any mode of inheritance (Munnich and Rustin, 2001). Additionally, dysfunction can originate from non-genetic causes as environmental influence (Munnich and Rustin, 2001).

In the last few years, it has become increasingly clear that defects of oxidative phosphorylation account for a large variety of clinical symptoms in both childhood and adulthood. mtDNA deletions and mutations at least among pediatric cases account for not more than 10-15 % of patients (Leonard and Schapira, 2000). Thus, in most cases, nuclear gene defects are presumably responsible for respiratory chain deficiency. Disease-causing nuclear gene mutations fall in two categories: 1) mutations in nuclear-encoded respiratory chain subunits, and 2) mutations in

assembly and maintenance protein genes (Munnich and Rustin, 2001). Surprisingly, only a small number of catalytic subunit gene mutations have been reported (genes for complex II and complex I). Moreover, systematic sequence analysis of nuclear encoded subunits of complex IV has failed to detect any mutation in their coding sequences until this year (first case was reported by Zeviani M. et al. in March 2008 at the *MITOCOMBAT meeting*). Taken together, these data support the view that mitochondrial assembly and maintenance are primarily altered in respiratory chain deficiencies (Munnich and Rustin, 2001).

Mitochondrial disorders affect at least 1 in 5000 individuals (Schaefer et al., 2004). Therapy of mitochondrial encephalomyopathies (as the defects of the mitochondrial respiratory chain are commonly named) is woefully inadequate, despite great progress during the past years in understanding of the molecular bases of these disorders. Palliative therapy is dictated by good medical practice and includes anticonvulsant medication, control of endocrine dysfunction, and surgical procedures (DiMauro and Mancuso, 2007; Zeviani and Di Donato, 2004). Lactic acidosis is one of the laboratory hallmarks of mitochondrial diseases. Pharmacological removal of this noxious metabolite extends to other metabolites as well (DiMauro et al., 2006). Administration of metabolites and cofactors is the mainstay of real-life therapy and is especially important in disorders due to primary deficiencies of specific compounds, such as carnitine or coenzyme Q_{10} (DiMauro and Mancuso, 2007). Aerobic exercise and physical therapy prevent or correct deconditioning and improve exercise tolerance in patients with mitochondrial myopathies due to mtDNA mutations (DiMauro and Mancuso, 2007). Gene and germline therapy is a challenge but it raises ethical problems. The accurate diagnosis is invaluable for the clinician and the patient, allowing prognostic and genetic counseling, and alerting the physician to potential complications in the future (Thorburn and Dahl, 2001). Even there is not a lack of treatment there is lack of cure, therefore preventive therapy through genetic counseling and prenatal diagnosis is highly important. The possibility of prenatal diagnostic is complicated by the dual origin of the mitochondrial proteins and is dependent on the knowledge of exact mutation in the affected family, while so far the proper prenatal diagnostic can be managed only in nuclearly encoded defects (Amiel et al., 2001; Faivre et al., 2000; Niers et al., 2003; Robinson, 2001).

COMPLEX IV – cytochrome c oxidase

Cytochrome c oxidase (CcO; COX) is the terminal component of respiratory chain and is embedded in the inner mitochondrial membrane together with the other proteins of respiratory chain (Barrientos et al., 2002a; Taanman and Williams, 2001). It is a place where electrons end its pilgrimage through the respiratory chain enzymes to find its final acceptor. CcO accepts electrons from reduced cytochrome c and uses these to reduce molecular oxygen to water. At the same time, CcO contributes to the generation of the mitochondrial proton gradient by moving protons across the membrane (Kadenbach et al., 2000; Taanman and Williams, 2001).

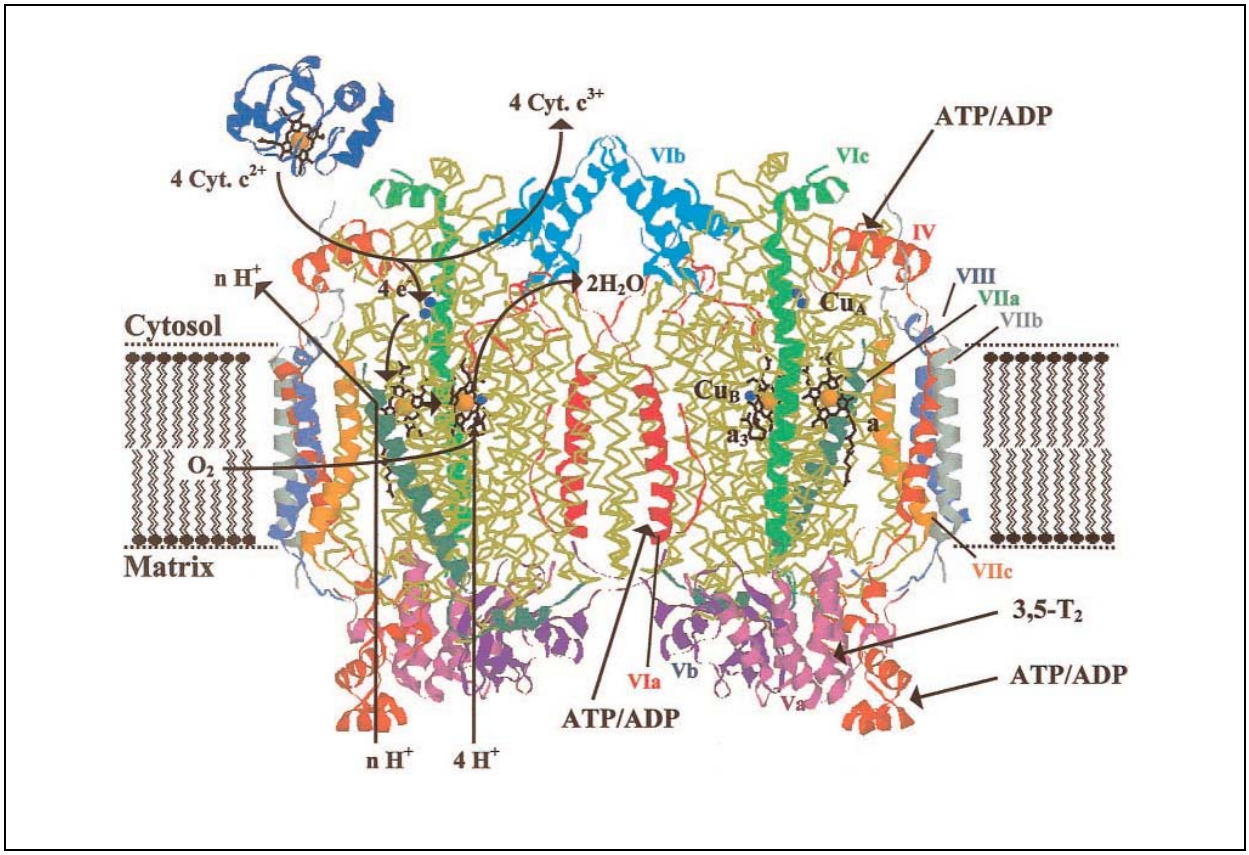
In mammals, CcO is present in the inner mitochondrial membrane as a large dimeric unit. Each monomer of the mammal protein consists of 13 subunits, similarly to 13 subunits found in the CcO from tuna (Arnold et al., 1997) and turkey (Kadenbach et al., 2000). Whereas, the CcO fulfils the same role in most of the organisms, the number of assembled subunits differs: 12 in yeast (Herrmann and Funes, 2005) or 7 in Dictiostelium discoideum (Kadenbach et al., 2000).

The crystal structure of the bovine enzyme solved by (Tsukihara et al., 1996) allowed detailed insights into the three-dimensional organization of the complex (see fig.3)

The three largest subunits, encoded by mtDNA, transcribed and translated in the mitochondrial matrix (Rodeheffer and Shadel, 2003), are the most evolutionary conserved (Ludwig et al., 2001). They form the core of the complex where the catalytic function relies on the redox-active catalytic cofactors including heme *a* and copper (Tsukihara et al., 1995, 1996). Subunit 1 (Cox1; COX I; CO I etc.) is the largest and most conserved one. It is composed of twelve transmembrane helices connected by short extramembrane loops and shows highly hydrophobic manners (Hermann and Shaw, 1998). Its catalytic function is connected to the protein translocation pathways (Stiburek et al., 2006) and to the two heme *a* moieties. The hemes are referred also as *a* and a_3, based on the spin states of their iron atoms. It explains one of the traditional names of CcO - cytochrome aa_3. Subunit 1 contains also one copper atom that form Cu_B site. Two other copper atoms form the binuclear Cu_A site of subunit 2 (Cox2; COX II; CO II etc.), which constitutes the docking site for cytochrome c (Stiburek et al., 2006). In addition, there is a magnesium ion occupying the cleft formed by subunits 1 and 2 (Tsukihara et al., 1995). Subunit 3 (Cox3; COX

III; CO III etc.) does not bear prosthetic group and its function is more in stabilization and correct functioning of the two others.

Fig. 3. Crystal structure of the dimeric cytochrome *c* oxidase from bovine heart, as located in the inner mitochondrial membrane bilayer. (Kadenbach et al., 2000)
On the cytosolic side, the crystal structure of a cytochrome *c* molecule (Cyt *c*) is shown in dark blue ribbons. Marked in yellow backbone are subunits I, II and III, the nuclear coded subunits are shown as ribbons in the indicated color. The hemes c, a, and a3 are indicated in black; the iron atoms in orange; and CuA and CuB in blue. On the left monomer, the chemical reaction is indicated schematically. On the right monomer, the nuclear-coded subunits are denoted, and the binding sites for the regulatory compounds ATP or ADP and 3,5-diiodothyronine (3,5-T2) are indicated.



The remaining 10 subunits are encoded in the nuclear genome and, after translation on ribosomes in cytoplasm, they are imported into the matrix through the membrane transport proteins (TIM and TOM complexes) (Pfanner and Wiedemann, 2002). Once imported, the nuclear-encoded subunits associate with the surfaces of subunits 1, 2, and 3 and form the CcO holoenzyme. These peripheral subunits have associated zinc and sodium ions, as well as several phospholipid molecules, but about their exact function is known very little.

The nuclearly encoded subunit IV of the bovine heart enzyme contains two specific ATP binding sites. One located at the cytosolic domain (Napiwotzki and Kadenbach, 1998) and the other at the matrix domain (Arnold and Kadenbach, 1997)

(see fig.2). Recent studies show the effect of ATP bound from the cytosolic side on the decrease of the affinity to cytochrome c of the bovine heart enzyme (Napiwotzki and Kadenbach, 1998). The findings leaded to the second theory of controlling mechanism of respiratory chain activity known also as allosteric inhibition (Kadenbach et al., 2000; Ludwig et al., 2001). The subunit IV plays also a crucial role in assembly processes of the whole CcO (Li et al., 2006).

Subunit Va contain a matrix side located specific binding place for 3,5-diodothyronine. Filling of this site prevent the allosteric inhibition of activity by ATP (Arnold et al., 1998). This mechanism could explain the well-known short-term effect of thyroid hormones on the gene expression independent resting metabolism of animals (Kadenbach et al., 2000; Soboll, 1993).

Protein kinase A binding site was located in the VIb subunit, which is located at the matrix site of the mitochondrial inner membrane (Yang et al., 1998). Its role in allosteric ATP-inhibition is connected with cAMP-depedent phosphorylation (Bender and Kadenbach, 2000).

In mammals, subunit VIa is expressed in two tissue-specific isoforms – the VIaL is induced permanently and the VIaH is induced at high intramitochondrial ATP/ADP ratios (Grossman and Lomax, 1997). VIaH is expressed exclusively in heart and skeletal muscle of mammals, and depending on the intramitochondrial ATP/ADP ratio can regulate the H^+/e^- stoichiometry from 1,0 to 0,5 (Anthony et al., 1993). The decrease probably participate in thermogenesis of mammalian skeletal muscle at rest when the ratio is high (Kadenbach et al., 1995). The VIaL isoform is expressed in all nonskeletal muscle tissues of mammals, where the H^+/e^- values are below 0,5 and are independent of the ATP/ADP ratios (Huttemann et al., 1999). Therefore, VIaL might participate in the permanent thermogenesis of warm-blooded mammals (Kadenbach et al., 2000).

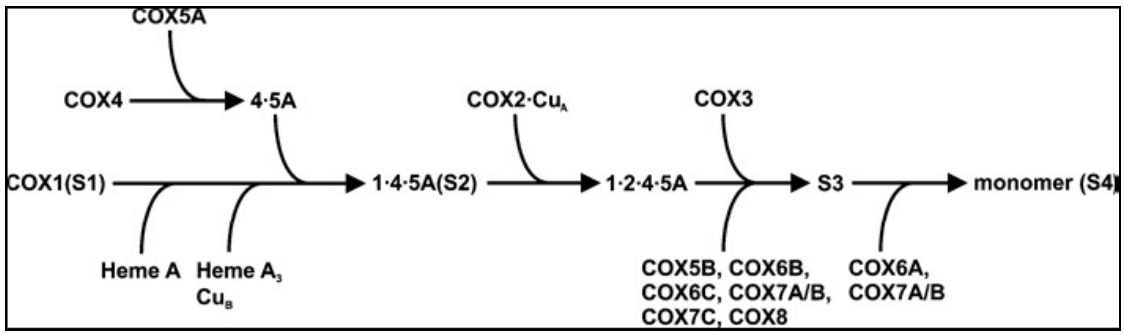
Similarly, the VIIaH isoform was found expressed in bovine heart and skeletal muscle, while in all other tissues, the VIIaL isoform was presented (Anthony et al., 1990). The exact function of the two isoforms is unknown, as well as the function of specific isoforms of subunit VIII – VIIIH; VIIIL, which were found in bovine, dog and rat enzyme, whereas in human, sheep and rabbit is expressed only one of them (Kadenbach et al., 2000; Linder et al., 1995).

The function of the other nuclearly encoded subunits is still remaining almost unknown; it is believed they mainly participate in stabilization of the whole CcO enzyme.

Over the years a great deal has been learned about the structure and arrangement of the subunits, but despite these advances, the manner, in which the enzyme is assembled in mitochondria, is still just partially understood (Fontanesi et al., 2006).

The correct assembly of all CcO components in the right order to the exact position is clearly a multistep, complicated and well organized process. Over 30 accessory nuclear-encoded proteins are involved. These auxiliary proteins play a key role in the formation of functional enzyme complex. The biogenesis of functional CcO enzyme occurs in several steps characterized by the formation of various assembly intermediates or subcomplexes (Nijtmans et al., 1998; Zee and Glerum, 2006).

Scheme 1. Proposed model of the assembly pathway of human COX (Stiburek et al., 2005)
The Arabic numerals denote subunits of the enzyme. S1–S4 indicate previously identified assembly intermediates. Prosthetic groups are also indicated. Dimerization of the 13-subunit holoenzyme (S4) completes the assembly of the COX complex.



The biggest approach was done by studies with COX-deficient human cells, where the existence of three main assembly intermediates was suggested (Nijtmans et al., 1998; Stiburek et al., 2005). In both yeast and humans, the three mitochondrially encoded subunits are transcribed and translated in the mitochondrial matrix with the assistance of several transcription, translation and assembly nuclearly encoded factors (Zee and Glerum, 2006). Formation of Cox1 subunit accompanied with heme *a* biosynthesis and incorporation, as well as with copper delivery, corresponds to the first assembly intermediate (S1). The second intermediate (S2) is formed by connection of the Cox1 with little complex formed from nuclearly encoded

subunits IV and Va. The third intermediate (S3) contains all of the subunits except subunits VIa and VIIa or VIIb. Addition of the last subunits leads to the formation of the holoenzyme monomer (S4) (Scheme 1.) (Fontanesi et al., 2006; Nijtmans et al., 1998; Stiburek et al., 2005; Ugalde et al., 2002).

All the studies are showing a huge group of additional functional proteins involved in different stages of the assembly processes, for example in translocation of nuclear-encoded subunits across the mitochondrial membranes, while many of them are highly hydrophobic; or in the formation and insertion of heme *a*, as well as in transport and embedding of primary high cell-toxic, but for correct function essential, ions of copper, zinc and magnesium into the nascent enzyme complex (Carr and Winge, 2003). However, understanding of all the pathways is in spite of all research still rumbling in the yeast, the situation in mammals is even more unclear. Studies in mammals described several human homologs of yeast assembly factors (see tab.1). Closer studies show some differences in their function and behaving from yeast ones (Zee and Glerum, 2006).

Tab 1. Functional classes of COX assembly factors. (Zee and Glerum, 2006)

Assembly factor	Reference	Function	Localization	Human homolog
Translational activators or regulators				
Pet309p	(Manthey and McEwen, 1995)	Cox1p translation	Matrix	LRPPRC
Mss51p	(Barrientos et al., 2004)	Regulation of Cox1p translation	Matrix	
Cox14p	(Barrientos et al., 2004)	Regulation of Cox1p translation	IMM	
Pet111p	(Mulero and Fox, 1993)	Cox2p translation	Matrix	
Pet54p	(Brown et al., 1994)	Cox3p translation	Matrix	
Pet122p	(Brown et al., 1994)	Cox3p translation	Matrix	
Pet494p	(Brown et al., 1994)	Cox3p translation	Matrix	
Membrane insertion				
Oxa1p	(Hell et al., 1998)	Insertion of charged domains	IMM	OXA1
Mba1p	(Ott et al., 2006)	Insertion of uncharged domains	IMM	
Cox18p	(Saracco and Fox, 2002)	Cox2p C-terminus insertion	IMM	
Mss2p	(Saracco and Fox, 2002)	Cox2p C-terminus insertion	IMM	
Pnt1p	(Saracco and Fox, 2002)	Cox2p C-terminus insertion	IMM	
Subunit-specific chaperones				
Cox20p	(Hell et al., 2000)	Cox2p chaperone	IMM	COX20
Copper insertion				
Cox17p	(Glerum et al., 1996a)	Copper chaperone	Cytosol/IMS	COX17
Cox11p	(Carr et al., 2005)	Copper insertion into Cox1p	IMM	COX11
Sco1p	(Dickinson et al., 2000)	Copper insertion into Cox2p	IMM	SCO1 /SCO2

Heme biosynthesis				
Cox10p	(Tzagoloff et al., 1993)	Heme B to heme O conversion	IMM	COX10
Cox15p	(Barros et al., 2001)	Heme O to heme A conversion	IMM	COX15
Assembly chaperones				
Shy1p	(Mashkevich et al., 1997)	Subcomplex assembly	IMM	SURF1
Pet100p	(Church et al., 2005)	Nuclear-encoded subunit assembly	IMM	
Unknown				
Cox19p	(Nobrega et al., 2002)	?	Cytosol/IMS	COX19
Cox23p	(Barros et al., 2004)	Copper chaperone?	Cytosol/IMS	COX23
Pet117p	(McEwen et al., 1993)	?	?	
Pet191p	(McEwen et al., 1993)	?	?	PET191
Cox16p	(Carlson et al., 2003)	?	IMM	COX16

Note: Question marks indicate that the information is unknown.
IMM for inner mitochondrial membrane

Beside the COX well known function in coupling of the electron transfer between cytochrome c and molecular oxygen, and vectorial translocation of protons across the inner mitochondrial membrane, the previously mentioned data predicts CcO to play an important role in regulation of all the processes of oxidative phosphorylation.

The respiratory control is generally understood as stimulated by ADP followed by its decrease due to conversion into ATP (Ludwig et al., 2001). This phenomenon could be explained by the chemiosmotic hypothesis (Mitchell, 1966).

The second system of regulation is based on phosphorylation of CcO by mitochondrial (cAMP-dependent) protein kinase, which turns on the allosteric inhibition of CcO at high intramitochondrial ATP/ADP ratios (Ludwig et al., 2001). Extracellular signals that increase mitochondrial Ca^{2+} are suggested to activate mitochondrial Ca^{2+}-dependent protein phosphatases and dephosphorylate cytochrome c oxidase (Kadenbach et al., 2000).

Human cytochrome c oxidase in health and disease

It is obvious that CcO plays irreplaceable role in the elementary process of cell. Just slight disruption in its structure may have devastating consequences for the whole process of energy supply of the cell, organ and whole organism. Mitochondrial diseases caused by respiratory chain disorders as mentioned previously are not as rare as commonly believed. Their estimated prevalence of 1 cases per 5,000 persons is similar or even higher to that of better known neurological diseases, such as

amyotrophic lateral sclerosis and the muscular dystrophies (Chinnery and Turnbull, 2001; Schaefer et al., 2004).

Isolated CcO deficiency, commonly named COX deficiency, represents one of the most frequent reasons of respiratory chain defects in humans associated with a wide spectrum of clinical phenotypes (Bohm et al., 2006; Shoubridge, 2001). Predominantly, there are affected tissues with high energetic demand, especially the brain, skeletal muscle and heart (DiMauro and Schon, 2003; Robinson, 2000). The clinical phenotype is usually severe and often fatal. As mentioned before, the therapy of mitochondrial diseases in general is still woefully inadequate (Zeviani and Di Donato, 2004) and genetic counseling with eventual prenatal diagnosis in affected families is complicated by several difficulties in searching for the basic cause of the disease. COX disorders can have one of two genetic origins: defects stemming from mutations in the mtDNA are either maternally inherited or sporadic, whereas defects encoded in the nuclear DNA will be inherited in an Mendelian fashion (Zee and Glerum, 2006). Only the clear understanding of basic pathogenic mechanisms based on a detailed characterization of COX deficiency at the protein level in various tissues and accompanied by molecular analyses, can enable the accurate genetic (Vesela et al., 2004).

Since the first large-scale deletion in mtDNA causing mitochondrial myopathy was described (Holt et al., 1988), many other mutations were reported in association to isolated COX deficiency or COX deficiency together with decrease of another respiratory chain enzyme (Keightley et al., 1996; Schon et al., 1989; Zeviani and Carelli, 2003). But mtDNA mutations explain only a minority of human COX deficiencies (Shoubridge, 2001). Based on family genealogies, it is assumed that most of the COX defects have its origin in nuclear genes. The main interest was concentrated to the screening of the 10 nuclear-encoded COX subunits, but until very recent no mutation has been found (Barrientos et al., 2002a; Coenen et al., 2006). The first case was presented by Zeviani et al. at the *MITOCOMBAT meeting* in March 2008. Therefore, the most non-maternally inherited mutations for COX defects must occur in non-structural genes (Zee and Glerum, 2006). The past decade has seen the identification of mutations in 6 COX assembly factors – *SURF1, SCO2, SCO1, COX10, COX15, and LRPPRC*.

Leigh syndrome and SURF1

Surf1 deficiency is the most common recognized cause of systemic cytochrome c oxidase deficiency. Although the biochemical defect is expressed in all aerobic tissues, clinical consequences are usually restricted to the function of central nervous system, and most patients present with Leigh disease (Head et al., 2004).

Leigh syndrome is an early-onset progressive neurodegenerative disorder with a characteristic neuropathology consisting of focal, bilateral lesions in one or more areas of the central nervous system, including the brainstem, thalamus, basal ganglia, cerebellum, and spinal cord. The lesions are areas of demyelization, gliosis, necrosis, spongiosis, or capillary proliferation. Clinical symptoms depend on which areas of the central nervous system are involved, but commonly include failure to thrive, feeding difficulties and hypotonia. The main laboratory findings are raised lactate in the blood and cerebrospinal fluid, but the diagnosis is confirmed only by the presence of bilateral symmetrical lesions (Rahman et al., 1996). Leigh syndrome can result from a number of different defects in mitochondrial energy metabolism, most commonly deficiencies of cytochrome oxidase (COX), pyruvate dehydrogenase, NADH-ubiquinone oxidoreductase (Complex I) and ATP synthase. In a small number of cases (Oquendo et al., 2004) with Leigh syndrome and COX deficiency, mutations in mitochondrial DNA (mtDNA) were found and Leigh syndrome was observed in some patients with mutations in the COX10 and COX15 gene (Antonicka et al., 2003a; Bugiani et al., 2005; Oquendo et al., 2004). But most of the patients with Leigh syndrome and cytochrome c oxidase deficiency are harbouring a mutation in the *SURF1* gene. There were described also two cases of patients with mutation in SURF1 gene but without Leigh syndrome phenotype (Salviati et al., 2004; Von Kleist-Retzow et al., 2001).

SURF1 in brief:

Acronym for: surfeit locus protein 1

Yeast homolog: *SHY1*

Human gene:

Position: chromosome 9q34

Structure: 9 exons spanning 4.7kb; on reverse strand

Transcript: 1037bps

Protein:

Structure: 300 residues; matured 30kD; 2 transmembrane domains

Localization: inner mitochondrial membrane

Function: unknown exactly – participate in maturation and expression of Cox1 due to R.sphaeroides insertion of active site heme a

Mutations: about 40 different (missense, nonsense, insertions and deletions affecting usually the C terminus disabling insertion of the protein to the inner mitochondrial membrane); 845_846delCT prevalent in Slavonic population

Clinical phenotype: mostly classical Leigh syndrome - subacute necrotizing encephalomyelopathy characterized by bilaterally symmetric necrotic lesions in subcortical brain regions. Von Kleist-Retzow et al. 2001 and Salviati et al. 2004 described two cases with isolated COX deficiency due to *SURF1* mutations but without typical Leigh syndrome phenotype.

Patient prognoses: poor due to the progressive fatal course; survival 3-8 years, rarely more

Described cases: many – Leigh syndrome is one of the most common disorders of the respiratory chain in infancy and childhood and mutations in *SURF1* explain the molecular background in a large proportion of these patients

(Barrientos et al., 2002b; Bohm et al., 2006; Brown and Brown, 1996; Head et al., 2004; Mashkevich et al., 1997; Munaro et al., 1997; Pecina et al., 2003; Pequignot et al., 2001; Salviati et al., 2004; Stiburek et al., 2006; Tiranti et al., 1999; Tiranti et al., 1998; Von Kleist-Retzow et al., 2001; Williams et al., 2001b; Yao and Shoubridge, 1999; Zee and Glerum, 2006; Zhu et al., 1998)

LRPPRC

Merante in 1993 reported the results of biochemical investigations on a group of patients from the Saguenay-Lac-Saint-Jean region of Quebec, who had an unusual form of cytochrome oxidase deficiency and Leigh disease (Merante et al., 1993). This group was distinguished from the classical presentation of COX deficiency with Leigh disease, by the severity of the biochemical defect in different tissues. The activity of COX in skin fibroblasts, amniocytes, and skeletal muscle was 50% of normal; in brain and liver, there were very low activities, and in kidney and heart it was close to normal values (Merante et al., 1993). Morin in 1993 presented the detail clinical and metabolical report in these children (Morin et al., 1993).

Mootha in 2003 took up the previous studies, which marked a *LRPPRC* gene on chromosome 2p16 as a candidate gene for Leigh syndrome, French-Canadian type (LSFC), and in 22 patients with LSFC phenotype identified mutations. 21 of them carried homozygous mutation c.1119C>T in exon 9 predicting a missense change A354V (Mootha et al., 2003). The last patient carried this mutation on one allele; while on the other allele, there was 8 nucleotides deletion in exon 35 resulting in a premature stop codone (Mootha et al., 2003). According to their results and previously published data, they suggested that *LRPPRC* participates probably in mRNA processing both in the nucleus and the mitochondrion.

LRPPRC in brief:

Acronym for: leucine-rich PPR motif-containing protein

Yeast homolog: PET309 - gene required for the stability and processing of Cox1p

Human gene:

Position: chromosome 2p21-p16

Structure: 38 exons spanning 128.26kb; on reverse strand

Transcript: 5095 bps

Protein:

Structure: 1394 residues; 130 kD; leucine-rich protein

Localization: mitochondrial matrix

Function: unknown, lower levels associated with reduced levels of COX mRNA transcripts within the mitochondria

Mutations: 21 out of 22 patients harbouring in exon 9 c.1061C>T (A354V) on both alleles, one patient is compound heterozygous for previous mutation and 8bp deletion in exon 35

Clinical phenotype: the Saguenay-Lac.St. Jean form of COX deficient Leigh syndrome called also Leigh syndrome – French Canadien type (LSFC) presenting together with characteristic changes of Leigh syndrome in the central nervous system also microvesicular steatosis, which corresponds well with almost absent COX activity in liver

Patient prognoses: poor due to the progressive fatal course; survival 6 months to 6 years, rarely up to 11 years

(Merante et al., 1993; Mootha et al., 2003; Morin et al., 1993; Xu et al., 2004)

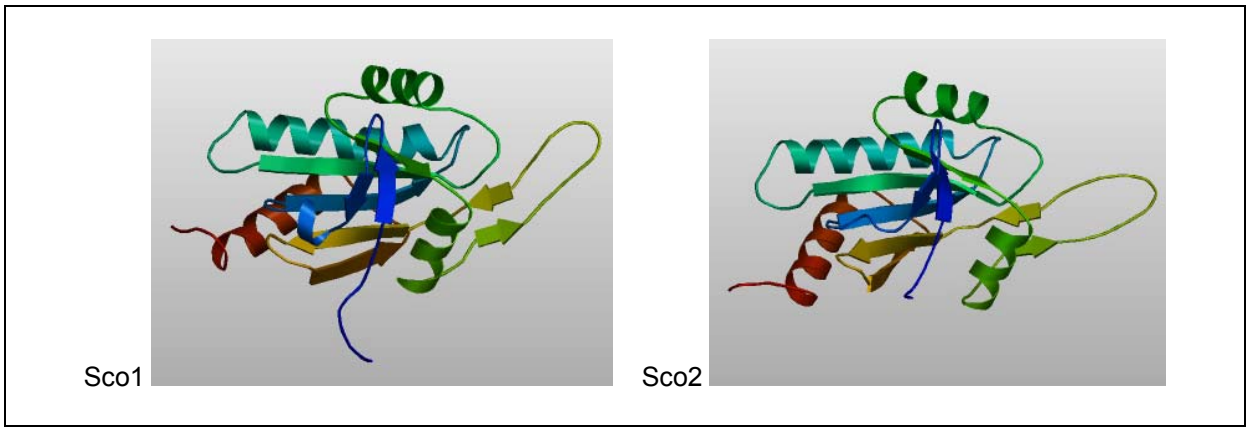
SCO protein family

Sco (an acronym for synthesis of cytochrome c oxidase) protein family in humans includes Sco1 and Sco2 proteins (Paret et al., 1999). Both Sco proteins are highly similar, and both are required for viability. These two proteins contain a highly conserved CxxxC motif that presumably binds copper, and therefore is hypothesized to transport copper to COX (Carr and Winge, 2003; Horng et al., 2005; Papadopoulou et al., 1999). The structural similarity to the thioredoxin family has led to the proposal that Sco functions as a thiol:disulfide oxidoreductase, instead of, or in addition to, a copper trafficking (Chinenov, 2000). On the basis of extreme sensitivity to hydrogen peroxide, the Sco proteins have also been proposed to function as copper-dependent redox switches in signaling (Williams et al., 2005). Due to the latest findings, Sco proteins seem to participate also in regulation of cellular copper homeostasis (Leary et al., 2007). The studies in yeast (Nittis et al., 2001) have a limited comparative value, while even sharing great identity in the core region (Cu binding site) with the human Sco polypeptides (Papadopoulou et al., 1999), their behaving is not identical. Yeast Sco2 seems to lack the essential role in COX assembly, although it is able, when overexpressed, to partially rescue Sco1 mutants (Briere and Tzagoloff, 2007). In contrast, patients with mutation in either Sco1 or Sco2 present a COX deficiency that is not rescuable by the respective homolog, indication nonoverlapping activities of the two proteins in metallation of subunit II (Glerum et al., 1996b; Leary et al., 2004). To explaine the current data Leary et al 2007 (Leary et al., 2007) proposed a model in which human Sco proteins cooperate on two separate aspects of copper metabolism. One is to promote maturation of the CuA site and the second is to regulate copper efflux under condition of excessive cellular copper. To date, many functional studies including structural characterization of human Sco2 (Banci et al., 2007a) and crystallization of yeast and human Sco1 proteins were done(Abajian and Rosenzweig, 2006; Balatri et al., 2003; Williams et al., 2005), however, the precise function of Sco proteins remains unclear.

Mutations in *SCO1* causing COX deficiency were described so far only in two boys from one family (Valnot et al., 2000a). First boy of unrelated healthy parents (birth weight 2700g, length 45cm) was born hypotonic and lethargic requiring immediate assistance for respiratory distress. Biochemical investigation revealed severe metabolic acidosis with abnormally high urinary lactate, fumarate and succinate. Severe axial hypotonia, hypoglycemia and hyperlactatemia together with

liver enlargement and recurrent episodes of apnea and bradycardy were observed to the age of 2 months when he died. Isolated COX deficiency was found in liver and skeletal muscle. One year later, a second boy was born (birth weight 1750g), who presented severe neurological distress and metabolic acidosis and died at the age of 5 days. Both patients were compound heterozygotes for c.363_364delGA, which results in a frameshift and premature codon in exon2, and c.520C>T changing a highly conserved praline into a leucine (P174L) (Valnot et al., 2000a) Its pathology was described by Banci et al (Banci et al., 2007b). And this mutant genotype was found to have an affect on the COX assembly (Paret et al., 2000) as well as on function of Cox17, another copper chaperone (Cobine et al., 2006a)

Fig 4. Structure models of proteins of SCO family showing high structural similarities (pfam)



Mutations in *SCO2* were mostly associated with fatal infantile hypertrophic cardiomyopathy (Jaksch et al., 2001a; Jaksch et al., 2000; Papadopoulou et al., 1999; Sacconi et al., 2003; Salviati et al., 2002b; Sue et al., 2000). The predominant symptoms were muscular hypotonia, severe hypertrophic cardiomyopathy (HCM), encephalopathy, peripheral neuropathy and a pronounced COX deficiency in striated muscle and most probably in the CNS (Freisinger et al., 2004). By that time we published group of 7 patients, where two distinct phenotypes due to the mutation type were presented. One with severe hypertrophic cardiomyopathy connected with compound heterozygous phenotypes and the other associated with homozygozity of g.1541G>A mutation with a bit milder clinical manifestation missing severe cardiologic involvement (Vesela et al., 2004). The g.1541G>A mutation was found at least on one allele in all described patients, so far. Therefore it is thought to be prevalent. There are studies showing the biochemical benefit of copper

supplementation in cultured cells from patients with Sco2 mutations (Jaksch et al., 2001b; Salviati et al., 2002a), but the progression in clinical manifestation in patients treated with copper was not observed (Jaksch et al., 2001a)

SCO in brief:

Acronym for: synthesis of cytochrome c oxidase

Yeast homolog: Sco1p

Function: Both human Sco proteins are highly similar, and both are required for viability. These two proteins contain a highly conserved CxxxC motif that presumably binds copper, thus it is hypothesized to transport copper to COX (Carr and Winge, 2003). The structural similarity to the thioredoxin family has led to the proposal that Sco functions as a thiol:disulfide oxidoreductase, instead of, or in addition to, a copper trafficking (Chinenov, 2000). On the basis of extreme sensitivity to hydrogen peroxide, it has also been proposed that Sco proteins function as copper-dependent redox switches in signaling (Williams et al., 2005). In the latest findings, Sco proteins seem to also participate in the regulation of cellular copper homeostasis (Leary et al., 2007). To date, however, the precise function of Sco proteins remains unclear.

SCO1:

Human gene: SCO1 (homologue 1)

Position: chromosome 17p13.1

Structure: 6 exons spanning 17.19 kb; on reverse strand

Transcript: 1708 bps

Protein:

Structure: 301 residues; 34 kD;

Localization: inner mitochondrial membrane

Mutations: 2-bp deletion, missense

Clinical phenotype: encephalopathy, myopathy, hepatopathy and cardiomyopathy

Patient prognoses: poor due to progressive fatal course; survival 5 days to 2 months (Abajian and Rosenzweig, 2006; Balatri et al., 2003; Briere and Tzagoloff, 2007; Dickinson et al., 2000; Horvath et al., 2000; Petruzzella et al., 1998; Valnot et al., 2000a; Williams et al., 2005; Winge, 2003)

SCO2:

Human gene: SCO2

Position: chromosome 22q13.33

Structure: 2 exons spanning 2.01kb; on reverse strand

Transcript: 992bps

Protein:

Structure: 266 residues; 30kD;

Localization: inner mitochondrial membrane

Mutations: g.1541G>A found in all patients at least on one allele; missense and short deletions (1 and 2bp)

Clinical phenotype: fatal infantile hypertrophic cardiomyopathy; encephalomyopathy; peripheral neuropathy; spinal muscular atrophy (in two cases)

Patient prognoses: poor due to the progressive fatal course; survival several days to 2 years

(Jaksch et al., 2001a; Jaksch et al., 2000; Leary et al., 2006; Papadopoulou et al., 1999; Petruzzella et al., 1998; Salviati et al., 2002b; Sue et al., 2000; Tarnopolsky et al., 2004; Tay et al., 2004)

Heme a synthesis

A heme is a metal-containing prosthetic group of several proteins, and consists of an iron atom embedded in a porphyrin ring system coordinated by four nitrogen atoms (Moraes et al., 2004). There are three biologically important forms of heme . types *a*, *b*, and *c* – which differ by modifications in the porphyrin ring. Most common type of heme is heme *b* (the prosthetic group of proteins like hemoglobin and myoglobin) and is also presented in the mitochondrial *bc1* complex, and it is not covalently bound to its apoprotein partner. Heme *c* contains two vinyl side chains and is covalently bound to the protein itself (cytochrome *c* and *c1*) (Moraes et al., 2004). Heme *a* is a unique heme compound present exclusively in COX. It is synthesized through a catalytic pathway that takes place inside mitochondria (Fontanesi et al., 2006). Heme *a* synthesized from heme *b* differs from that protoheme in that a methyl side chain is oxidized into a formyl group, and one of the vinyl side chains (on C2) is replaced by a isoprenoid (farnesyl) chain (Moraes et al., 2004). This process is catalyzed by several enzymes including products of *COX10* and *COX15* genes.

COX10

After detail studies of Cox10p in yeast, the human ortholog (*COX10*) was cloned and described as a candidate gene for COX deficiency in man (Glerum and Tzagoloff, 1994). Murakami described in 1997 the genomic structure and expression of the human *COX10* gene (Murakami et al., 1997). The gene encodes heme *a* farensyl transferase, which catalyzes the transfer of a farnesyl group to the vinyl at position 9 of the porphyrin ring system (Petruzzella et al., 1998). This reaction is the early one in the process of generation of heme *a* from protoheme (heme *b*). By the farnesylation of the heme *b* is produced heme O, the precursor molecule of heme *a* (Bugiani et al., 2005).

The firs patient, a boy with isolated COX deficiency born to first-cousin parents, was found homozygous for c.612C>A transversion in exon 4 of the COX10 gene. This transversion resulted in the change of an uncharged asparagine residue into a basic lysine residue (N204K). Both parents were heterozygous for the transversion. The boy had an unremarkable development until the age of 18 months, at which time he developed ataxia. At 2 years of age he presented with poor eye contact, severe muscle weakness, hypotonia, ataxia, ptosis, pyramidal syndrome, and status epilepticus. Increased urinary amino acids were suggestive of a proximal tubulopathy. Isolated COX deficiency in muscle, isolated lymphocytes, and cultivated fibroblasts was presented. He died at 2 years of age. An older sister had died at 5 years of age of a mitochondrial encephalopathy ascribed to COX deficiency. A younger sister had progressive neurologic deterioration with similar biochemical anomalies at the age of 2 years and died at the age of 3 years (Valnot et al., 2000b).

Three years later, Antonicka et al. 2003 (Antonicka et al., 2003a) described two other cases:

A white male infant born at term, presented in the first week of life with hypoglycemia, metabolic acidosis, hypotonia and poor feeding. Metabolic laboratory evaluation showed persistent lactic acidosis, elevated plasma alanine and proline, and no evidence of an organic acidemia. Profound sensorineural hearing loss was demonstrated by brainstem evoked response testing at 2 months age. Severe transfusion-dependent macrocytic anemia developed by 3 months. He was hospitalized with acute episodes of dehydration, metabolic acidosis and hypotension complicated by anemia. Cardiovascular evaluation revealed severe biventricular hypertrophic cardiomyopathy at age 4 months. Diagnostic muscle biopsy showed

normal structural histology but severely reduced or absent COX staining in most fibers. COX activity measured in mitochondria isolated from frozen muscle was less than 5% of control values; activities of all other respiratory chain enzymes were normal. He died at the age of 5 months. Molecular analyzes revealed c.791C> A transversion in exon 4, and a c.878C>T transition in exon 5 of the COX10 gene, predicting amino acid substitutions at T196K and P225L (Antonicka et al., 2003a).

The second patient, a girl, was the third child of healthy, non-consanguinous parents. Her development stopped suddenly after one and a half months and she presented with hypotrophy, transfusion-dependent anemia, slight splenomegaly, severe hypotonia and high lactate/pyruvate levels in both blood and CSF. MRI analysis showed severe symmetrical lesions typical for the Leigh syndrome. She died at the age of 4 months due to a central respiratory failure. COX activity in a muscle biopsy was decreased to 16% of the lowest control values and in fibroblasts down to 18%, while the other respiratory enzymes activities were normal. The patient was heterozygous for two missense mutations in exon 7, an c.1211A>T transversion and an c.1211A>G transition, predicting two different amino acid substitutions at the same evolutionarily conserved site in the protein: D336V and D336G (Antonicka et al., 2003a).

Last patient was described in 2004 by Coenen et al. (Coenen et al., 2004). The boy patient was born at term as the first child of consanguineous parents. At the age of 5 months, he developed progressive failure to thrive, and pronounced motor agitation was noted. Gross motor development was severely delayed at 7 months together with generalized muscular hypotonia with persistent head lag at traction, ataxia, hypermetria, exaggerated tendon reflexes with enlarged reflex zones, low amplitude nystagmus, and saccadic eye movements. Ocular fixation was weak. He was not able to grasp. Laboratory evaluation showed metabolic acidosis with elevated serum and cerebrospinal fluid lactate concentrations. Magnetic resonance imaging of the brain showed a pattern comparable to Leigh-like disease. Biochemical COX activity was significantly reduced in muscle and fibroblasts. The boy died at 9 months of age during acute respiratory infection due to cardiorespiratory failure (Coenen et al., 2004).

COX10 in brief:

Yeast homolog: Cox10p

Human gene:

Position: chromosome 17p12-p11.2

Structure: 7 exons spanning 139.27kb; on forward strand

Transcript: 2993bps

Protein:

Structure: 443 residues; 49kD

Protein family and names: protoheme IX farnesyltransferase, mitochondrial precursor; heme O synthase; heme *a* farnesyltransferase

Localization: inner mitochondrial membrane

Function: catalyzing of the farnesylation of a vinyl group at position C2 resulting in the conversion of protoheme (heme *b*) to heme O

Mutations: 6 different at 4 patients described so far

Clinical phenotype: progressive mitochondrial encephalopathy, proximal renal tubulopathy, Leigh-like syndrome, fatal infantile hypertophic cardiomyopathy, transfusiondependent anemia

Patient prognoses: poor due to the progressive fatal course; survival from 5 months to 5 years

(Antonicka et al., 2003a; Coenen et al., 2004; Glerum and Tzagoloff, 1994; Valnot et al., 2000b)

COX15

Studies on yeast mutants indicate that *COX15* is part of a three component mono-oxygenase, catalyzing the hydroxylation of the methyl group at position 8 of heme O, the precursor molecule of heme *a*. Heme O is the product of the farnesylation of protoheme, a reaction catalyzed by *COX10*. The alcohol group added to heme O is then oxidized into the corresponding aldehyde by an unknown enzyme, to produce heme *a* (Bugiani et al., 2005). The human *COX15* has two splice variants (*COX15.1* and *COX15.2* [GenBank accession numbers NM_078470 and NM_004376]) that differ in the predicted C-terminal amino acid sequence and 3´UTR (Antonicka et al., 2003b), but the functional role of the two isoforms remains to be established (Petruzzella et al., 1998).

The first patient with cytochrome c oxidase deficiency due to mutations in the COX15 was described in 2003 (Antonicka et al., 2003b). This patient developed soon after birth muscle hypotonia, epilepsy, and lactic acidosis, but the clinical course was

dominated by the development of a rapidly fatal hypertrophic cardiomyopathy. The patient died at the age of 24 days. The COX residual activity was markedly reduced in heart tissue (6%) and was low (25–30%) in liver, kidney, and cultured fibroblasts but normal in skeletal muscle. The child was a compound heterozygote with a missense mutation (c.700C>T, R217W) in exon 5 on one allele and a splice site mutation (c.447-3C>G) in intron 3 on the other allele, resulting in the deletion of exon 4 (Antonicka et al., 2003b).

Oquendo 2004 (Oquendo et al., 2004) reported the same c.700>RT missense mutation (R217W) in a homozygous form in a child with rapidly progressive Leigh syndrome and chronic gastrointestinal dysfunction. The first symptoms of the disease (hypotony, feeding difficulties and horizontal nystagmus) manifested at the age of 7 months. Sequentially, other symptoms were following – motor regression, progressive microcephaly and retinopathy. Magnetic resonance imaging of the brain at 1 year of age showed bilateral lesions in several regions compatible with a diagnosis of Leigh syndrome. He died at 4 years of age due to pneumonia. COX activity measured spectrophotometricaly in fibroblasts was under the detection level (Oquendo et al., 2004).

Last case, published so far, presents a patient with feeding difficulties and failure to thrive from the first days of life. Subsequently, he developed psychomotor delay, hypotonia, muscle weakness, cerebellar tremor, and eye movement incoordination. MRI at 18 months of age diagnosed the Leigh syndrome. The symptoms slowly worsened thereafter with virtual body growth arrest, progressive loss of postural control. At the age of 4 years the MRI showed mild progression of the described changes, and in spite of unchanged clinical features, the plasma lactate and pyruvate previously markedly elevated decreased to normal levels. Apart from the central nervous system and skeletal muscle, the patient showed no abnormality in other tissues or organs. The boy was 16 years old and living in the time of the literature report. Biochemical assays showed an isolated COX deficiency both in skeletal Muscle and cultured fibroblasts (42% and 22% residual activity compared to the mean of controls, respectively). As a cause of his disease, there were found two novel heterozygous mutations in COX15 gene. The first mutation, a 503C>G transversion, introduces a premature stop codon in exon 4 (H152X). The second mutation, a 1081T>C missense transition in exon 8, results in a serine (highly conserved at this position among the species) to proline substitution at amino acid

position 344, in the C-terminal end of the fifth predicted transmembrane domain of the protein (Bugiani et al., 2005).

COX15 in brief:

Yeast homolog: Cox15p

Human gene:

Position: chromosome 10q24

Structure: 9 exons spanning 20.82kb; on reverse strand

Transcript: 4588bps

Protein:

Structure: 410 residues; 46kD; 2 isoforms varying in C termini (COX15.1 and COX15.2); 5 transmembrane domains; mitochondrial targeting sequence

Localization: inner mitochondrial membrane

Function: involved in catalyzing the hydroxylation of the methyl group at position 8 of heme O, the precursor molecule of heme *a*

Mutations: 3 different missense; one splicing mutation

Clinical phenotype: 3 cases so far, each with different phenotype: hypertrophic cardiomyopathy; rapidly fatal Leigh Syndrome; Leigh syndrome surviving at 16 years of age

Patient prognoses: poor due to the progressive fatal course; survival 23 days up to second decade

(Antonicka et al., 2003b; Bugiani et al., 2005; Oquendo et al., 2004; Petruzzella et al., 1998)

AIMS OF THE PRESENTED WORK

The laboratory for study of mitochondrial disorders works as a diagnostic center for patients from Czech and Slovak republics. During the last years, there were found over 40 children with isolated COX deficiency. Mutations in *SURF1* were found in 12 of them ((Bohm et al., 2006; Pecina et al., 2003) and unpublished cases).

Even the adequate treatment is unknown, the understanding of the illness causes is important not only for the physicians but as well for the families. The genetic counseling was offered to these families harbouring SURF1 mutations and several of them embrace the possibility of prenatal diagnostics.

Molecular background of the other cases was remaining unknown. Due to the possible dual origin of the defect with different hereditary aspects, the genetic counseling in the affected families was complicated and prenatal diagnostics very problematical if even possible. Previous experience leaded us to the decision to continue with searching for the molecular background in the remaining patients. This aim had to help not only with genetic counseling, but also in better treatment due to the better understanding of the etiology of the disease as well as to bring more information to clarify the metabolic processes in the context of cell energy generation.

The specific aims of this work were:

1. to select candidate genes for the screening in patients with biochemically confirmed COX deficiency
2. to optimize suitable methods for analyses
3. to search selected genes for mutations in the group of our COX deficient patients
4. to study the pathology of found mutations
5. to establishing of the screening methods to the routine diagnostic and bringing the possibility of the genetic counseling and relevant prenatal diagnostics

MATERIAL ANS METHODS

Ethics

The presented studies were carried out in accordance with the Declaration of Helsinki of the World Medical Association, and were approved by the Committees of Medical Ethics at Faculty of Medicine, Charles University and General Faculty Hospital in Praha. Informed parental consent, in accordance with guidelines of the participating institutions, was obtained.

Ad 1) to select candidate genes for our screening

Based on world published research data, we decided to screen the genes for COX assembly proteins with already described cases – both genes of the *SCO* protein family (*SCO2* and *SCO1)* and *COX10*. Later, in 2003, after the first published case, we added also *COX15* gene. We collected all available clinical and biochemical data of published patients and according to them we selected candidate patients with similar phenotype to analyze the certain gen.

The criteria were for:

- "*SCO2* group": isolated COX deficiency, lactic acidosis, rapid course of the disease, hypertrophic cardiomyopathy, encephalopathy, hypotony, respiratory failure, and life-span up to 2 years
- "SCO1 group": isolated COX deficiency, lactic acidosis, rapid course of the disease, hypotony, cardiological and liver involvement, and life-span up to 1 year
- "COX10 group": progressive mitochondrial encephalopathy, proximal renal tubulopathy, and a life-span up to 2 years; later also Leigh-like syndrome, fatal infantile hypertophic cardiomyopathy
- "COX15 group": at first - hypertrophic cardiomyopathy with several months surviving; later also rapidly fatal Leigh Syndrome; Leigh syndrome surviving and longer life-span

All groups were dynamic, due to the new patients and due to the literature news.

Ad 2) to optimize suitable methods

Molecular genetic methods

DNA isolation

As a material, patients DNA isolated from peripheral blood or from cultivated fibroblasts were used. Rarely, in case of no other choice, DNA was isolated from available tissue obtained at autopsy (skeletal muscle, brain, liver or heart tissue).

DNA isolation from peripheral blood was performed by common high-salt precipitation method. In case of small amount of blood and for isolation from other tissues, the QIAamp DNA Mini Kit (QIAGEN) was used, and all the isolation processes were performed according to the recommended manufacturer protocols.

Polymerase chain reaction

The primers were designed to the intronic regions surrounding the exons to be able to analyze also the neighboring regions of the coding sequences to avoid missing of splicing-site mutations. To the 5´end of the specific primers, sequence of a universal primer was added. So, all the specific sequencing reactions could be performed just with two universal primers (one forward and one reverse). Primer sequences used in our study are collected in tab.2

The amplification of searched fragments by polymerase chain reaction was optimized as well as the amplification of the sequencing reaction with the universal primers according to the needs of the sequencing analyzer. Optimizing and all amplifications were performed on MJ Research Thermal Cyclers.

To be able to confirm two mutations in *SCO2* gene, the restriction side had to be created. We used semi-nested PCR with a specific mismatch primer. As a template, we used PCR product of the first PCR, which amplified the specific fragment and than using one of the first-PCR-primer and a mismatch primer we performed second PCR. The mismatch primers were designed to create a specific restriction site for specific restriction enzyme to distinguish fragments with and without mutation by single RFLP analysis.

Tab.2 Primer sequences

Fragment	exons included	forward primer 5' →3' end	reverse primer 5' →3' end
universal primer	"RP"	caggaaacagctatgac	
universal primer	"T7"		aatacgactcactatag
SCO2		**NCBI -** AF177385	
fr1	1st part of 2nd	nt 1080 - nt 1098	nt 1386 - nt 1405
fr2	2nd part of 2nd	nt 1355 - nt 1375	nt 1778 - nt 1801
fr3	3rd part of 2nd	nt 1523 - nt 1543	nt 1979 - nt 1999
Mismatch primer	"1518delA"	nt 1487 - nt 1516; 1515C>G	
Mismatch primer	"1541 G>A"		nt 1543 - nt 1568; 1546G>T
SCO1		**NCBI - NT_010718**	
fr1	1st	RP+ nt 20 - nt 39	nt 414 - nt 433
fr2	2nd	nt 1589 - nt 1608	nt 1873 - nt 1894
fr3	3rd	nt 4530 - nt 4549	nt 4841 - nt 4860
fr4	4th	nt 5541 - nt 5560	nt 5815 - nt 5834
fr5	5th	nt 10668 - nt 10688	nt 10878 - nt 10894
fr6	6th	nt 16288 - nt 16303	nt 16579 - nt 16595
COX10		**NCBI - NC_000017**	
fr1	1st	nt 51 - nt 70	nt 402 - nt 421
fr2	2nd	nt 4841 - nt 4860	nt 5090 - nt 5111
fr3	3rd	nt 7244 - nt 7266	nt 7761 - nt 7787
fr4	4th	nt 32664 - nt 32673	nt 33000 - nt 33021
fr5	5th	nt 90345 - nt 90365	nt 90693 - nt 90713
fr6	6th	nt 122565 - nt 122585	nt 122852 - nt 122870
fr7a	1st part of 7th	nt 137287 - nt 137304	nt 137653 - nt 137672
fr7b	2nd part of 7th	nt 137402 - nt 137418	nt 137974 - nt 137993
COX15		**NCBI - NT_030059**	
fr1	1st	nt 20446 - nt 20465	nt 20617 - nt 20636
fr2	2nd	nt 17932 - nt 17949	nt 18432 - nt 18450
fr3	3rd	nt 15817 - nt 15835	nt 16280 - nt 16298
fr4	4th	nt 15481 - nt 15500	nt 15732 - nt 15751
fr5	5th	nt 12287 - nt 12304	nt 12791 - nt 12806
fr6	6th	nt 9405 - nt 9423	nt 9824 - nt 9833
fr7	7th	nt 6705 - nt 6725	nt 7252 - nt 7269
fr8	8th	nt 4724 - nt 4744	nt 5179 - nt 5197
fr9a	9.1	nt 3108 - nt 3124	nt 3348 - nt 3366
fr9b	9.2	nt 1884 - nt 1901	nt 2230 - nt 2248

PCR conditions and exact chemicals used for PCR in detail:

PCR elements:

1. self mix:
   a) KlenTaq polymerase (Gene Age Technologies, Czech Republic), stock concentration: 5units/ul
   b) pC2 buffer (Gene Age Technologies, Czech Republic- 50mM Tris, pH9,1; 16mM amonium sulphate; 3,5mM $MgCl_2$; 150 μg/ml BSA), stock concentration: 10x
   c) Taq polymerase - (Sigma; product No.: D6670), stock concentration: 5units/ul
   d) dNTP - Deoxynucleotide Mix (Sigma; product No.: D 7295), stock concentration: 10mM; working concentration: 2mM
   e) DMSO - Dimethyl sulphoxide (Sigma; product No.: D 2650)

f) $MgCl_2$ solution - (Sigma; product No.: M8787); stock concentration: 25mM
g) Primers - commercially synthesized according our sequence by Generi-Biotech company (working concentration 10pmol/ul)

2. commercially available kit:
a) Combi PPP Master Mix (Taq-Purple DNA Polymerase PCR Master Mix with MgCl and monoclonal antibody anti-Taq); Top-Bio s.r.o.; product No.: P-126)

SCO2:

All 3 fragments:

PCR reaction mixture:

element	*final concentration in 1 reaction (25μl)*
PC2 buffer	1x
dNTP	0,2mM
DMSO	6%
primer forward	0,4μM
primer reverse	0,4μM
Klen Taq polymerase	1,5 U
gDNA	50-100ng

PCR conditions:

	Step	*Temperature*	*Time*
1.	initial denaturation	94°C	2´
2.	denaturation	94°C	30´´
3.	annealing	65,4°C	30´´
4.	extension	72°C	1´
5.	28 times repeating steps 2 to 4		
6.	denaturation	94°C	1´
7.	annealing	65,4°C	30´´
8.	extension	72°C	3´

Seminested-PCR:

PCR of 2nd fragment for RFLP of g.1518delA using instead forward primer the delFm and for RFLP of g. 1541G>A instead of reverse primer the Bm one

PCR reaction mixture:

element	*final concentration in 1 reaction (25μl)*
PC2 buffer	1x
dNTP	0,2mM
$MgCl_2$	1,175mM
primer forward	0,4μM
primer reverse	0,4μM
Taq polymerase	1,5 U
PCR product fr2	5μl

PCR conditions:

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	2´
2.	denaturation	95°C	30´´
3.	annealing	54,6°C	15´´
4.	extension	72°C	40´´
5.	30 times repeating steps 2 to 4		
6.	final extension	72°C	7´

SCO1

PCR reaction mixture:

element	*final concentration in 1 reaction (25μl)*
PC2 buffer	1x
dNTP	0,2mM
DMSO	*
primer forward	0,4μM
primer reverse	0,4μM
Klen Taq polymerase	0,5 U
gDNA	50-100ng

* fr1 – 7%; fr4 – 6%; fr5 – 6%; fr6 – 10%

PCR conditions:

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	2´
2.	denaturation	95°C	30´´
3.	annealing	**	30´´
4.	extension	72°C	1´
5.	32 times repeating steps 2 to 4		
6.	denaturation	95°C	1´
7.	annealing	**	30´´
8.	extension	72°C	3´

** fr1, 3, 4, 6 = 57,3°C; fr2 = 53°C; fr5 = 54,4°C

COX10

All fragments excluding fr2:

PCR reaction mixture:

element	*final concentration in 1 reaction (25μl)*
PC2 buffer	1x
dNTP	0,2mM
DMSO	*
primer forward	0,6μM
primer reverse	0,6μM
Klen Taq polymerase	0,5 U
gDNA	50-100ng

* fr6 = 2%; fr7a = 6%; fr7b = 2%

PCR conditions:

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	2´
2.	denaturation	95°C	30´´
3.	annealing	**	30´´
4.	extension	72°C	1´
5.	32 times repeating steps 2 to 4		
6.	denaturation	95°C	1´
7.	annealing	**	30´´
8.	extension	72°C	3´

** fr1, 7a, 7b = 60,6°C; fr3, 4, 5, 6 = 58,4°C

Fragment 2:

PCR reaction mixture:

element	*final concentration in 1 reaction (25μl)*
PC2 buffer	1x
dNTP	0,2mM
$MgCl_2$	1,175mM
primer forward	0,6μM
primer reverse	0,6μM
Taq polymerase	1,5 U
PCR product	5μl

PCR conditions:

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	2´
2.	denaturation	95°C	30´´
3.	annealing	54°C	20´´
4.	extension	72°C	40´´
5.	30 times repeating steps 2 to 4		
6.	final extension	72°C	7´

COX15

All fragments excluding fr2:

PCR reaction mixture:

element	*final concentration in 1 reaction (25μl)*
CombiPPPMaster Mix	1x
DMSO	*
primer forward	0,4μM
primer reverse	0,4μM
gDNA	50-100ng

* fr8 = 4%

PCR conditions:

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	2´
2.	denaturation	95°C	30´´
3.	annealing	*	30´´
4.	extension	72°C	1´
5.	28 times repeating steps 2 to 4		
6.	denaturation	95°C	1´
7.	annealing	*	30´´
8.	extension	72°C	3´

**fr7= 64,8°C; all others = 59,5°C

Purification of PCR products

Amplified PCR products were extracted from the agarose gel (Sigma) and purified by gel extraction using QIAquick Gel Extraction Kit (QIAGEN) or Wizard SV

Gel and PCR Clean-Up System (Promega). All steps were performed according to the recommended manufacturer protocols. The gel extraction method turned out to have better results for sequencing analyzes than direct PCR products cleaning.

Sequencing

The first part of samples, most of the fragments screened for mutations in *SCO2*, was sequenced by gel automated cycle sequencing analyzer AlfExpress (Amersham Pharmacia Biotech) using AmpliTaq polymerase (Applied Biosystems) and RP, T7 universal cy5-labelled primers. The mixture for sequencing reaction contained 1xpC2 buffer; 4,8mM MgCl2; AmpliTaq polymerase according to the manufacturer; 0,3mM dNTPs containing 7-deaza-dGTP (Amersham Biosciences); 2,4μM ddNTPs; 0,14μMof cy5-labelled primer and 75ng of PCR template. The fragments for sequencing were prepared in steps see tab 3.

Tab 3. PCR conditions for sequencing

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	5´
2.	denaturation	95°C	5´´
3.	annealing	50°C	25´´
4.	extension	68°C	50´´
5.	35 times repeating steps 2 to 4		

Later a new capillary instrument, the ABI PRISM 3100-*Avant* Genetic Analyzer from Applied Biosystems, was available in the laboratory and the sequencing process was optimized for the new instrument. It simplified and speeded up the process, so most of *SCO1*, *COX10* and *COX15* fragments were analyzed on this new instrument. For the sequencing PCR reaction was used the chemistry of Big Dye Terminator v.3.1 Cycle Sequencing Kit according to the manufacturer protocol with slide modification in mixture. We used to the 20 μl of total reaction amount instead of 8 μl of commercial mixture kit only 2 μl and the buffer conditions were kept by adding of 3 μl of Big Dye Terminator v.1.1; 3.1 5x Sequencing Buffer (Applied Biosystems). 3,2 pmol of primer and concentration of template according to the recommended amounts, as well as the PCR conditions were kept due to the manufacturer protocols. Cleaning of sequencing reaction was carried out according to the recommended

ethanol precipitation protocols. For final denaturation, the Hi-Di Formamid (Applied Biosystems) was used in the amount of 20 μl per reaction. To guarantee perfect denaturation of all sequenced DNA regions, the ready reaction mixture dissolved in 20 μl of Hi-Di Formamide was denaturated by heating up to 95 °C for 3 minutes. For the sequencing the 50 cm or 80 cm capillary (Applied Biosystems) was used.

The obtained data were analyzed manually, later than by SeqScape Software v2.5 (AppliedBiosystems).

RFLP analyses

Found mutations were verified by a relevant PCR RFLP (restriction fragment length polymorphism) method (tab.4) according to the manufacturer protocols to each restriction endonuclease (buffer conditions, amount and temperature). The same method was later used also for screening of controls in the cases of novel (not described) mutations.

Tab 4. RFLP analysis

mutation	*restriction endonuclease*	*PCR fragment used for analyses*	*notes*
SCO2			
g.1182G>C.	Eco88I (Fermentas)	Fr. 1	Wild type - 0cut; mutated - 1cut
g.1280C>T	BfaI (BioLabs)	Fr. 1	Wild type - 0 cut; mutated - 1cut
g.1518delA	MaeIII (Roche)	Fr. 2 + Nested PCR with mismatch primer "1518delA"	Wild type - 2 cuts; mutated - 1cut
g.1541G>A	HindIII (Fermentas)	Fr. 2 + Nested PCR with mismatch primer "1541 G>A"	Wild type - 0 cut; mutated - 1cut
g.1756A>C	EcoO109 (Fermentas)	Fr. 2	Wild type - 1cut; mutated - 0cut
COX10			
c.184A>T	MaeIII (Roche)	Fr. 3	Wild type - 1cut; mutated - 0cut
c.476G>A	TaqI (Fermentas)	Fr. 3	Wild type - 1cut; mutated - 0cut
c.1291C>T	Eco52I (Fermentas)	Fr. 7b	Wild type - 1cut; mutated - 0cut
SCO1			
c.394G>A	HpaII (BioLabs)	Fr. 3	Wild type -0cut; mutated - 1cut

Biochemical methods*

Cell cultures and tissues

All studied tissues (blood, skeletal and heart muscle, liver, brain, kidney) and primary fibroblast cultures were obtained from three patients harbouring two different combinations of *SCO2* mutations, three patients carrying mutations in *SURF1* and

* these analyses were performed in collaboration with L. Stibůrek

age-related controls. Primary skin fibroblast cultures were established from forearm skin biopsy. Open muscle biopsies were obtained from the tibialis anterior muscle and were frozen at −80◦C. Post-mortem heart, liver, brain (basal ganglia) and kidney tissue specimens were removed and frozen less than 2 h after death.

Isolation of mitoplasts and mitochondria

Skeletal muscle, brain, heart and kidney mitochondria were isolated according to standard differential centrifugation procedures (Rickwood D, 1987) in a buffer comprising 150 mM KCl, 10 mM Tris/HCl, 2 mM EDTA and 2 *μ*g/ml aprotinin (pH 7.4) at 4°C. Liver mitochondria were isolated in a buffer comprising 250 mM sucrose, 20 mM Tris/HCl, 2 mM EDTA and 2 *μ*g/ml aprotinin (pH 7.4) at 4°C. Mitoplast-enriched fractions were prepared from cultured fibroblasts using digitonin (Sigma–Aldrich) as described in (Klement et al., 1995), with a final digitonin/protein ratio of 0.6 mg/mg. Protein concentrations were determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). All samples were stored at −80°C.

Enzyme activity assays

Activities of COX and CS (citrate synthase) were measured spectrophotometrically in fibroblasts and isolated tissue mitochondria essentially as described in (Rustin et al., 1994).

Electrophoresis

BN-PAGE (Blue Native PAGE) (Schagger and von Jagow, 1991) was used for separation of mitochondrial membrane protein complexes on polyacrylamide 8–15, 8–16 and 10–18% (w/v) gradient gels using a Mini Protean® 3 System (Bio-Rad Laboratories). Mitoplasts or mitochondria were solubilized with DDM (n-dodecyl *β*-D-maltoside; Sigma–Aldrich) with a final DDM/protein ratio of 1.0 mg/mg in a buffer containing 1.5 M aminocaproic acid, 2 mM EDTA and 50 mM Bis-Tris (pH 7.0) at 4°C. Serva Blue G (Serva) was added to solubilized protein at a concentration of 0.1 mg/mg of detergent, and 5–50 *μ*g of protein was loaded for each lane. The electrophoresis was performed at 40 V, 4°C for 1 h and then at 100 V, 4°C. Tricine SDS/PAGE was carried out under standard conditions with 12% polyacrylamide, 0.1% (w/v) SDS and 5.5 M urea gels. Mitochondrial fractions were dissociated in 50 mM Tris/HCl (pH 6.8), 12% (v/v) glycerol, 4% SDS, 2% (v/v) 2-mercaptoethanol and

0.01%(w/v) Bromophenol Blue for 30 min at 37°C, and approx. 10 *μ*g of protein was loaded for each lane. For two-dimensional BN/SDS/PAGE (Schagger and von Jagow, 1991), strips of the first-dimension gels were incubated for 40 min in 1% 2-mercaptoethanol and 1% SDS and then for 10 min in 1% SDS, and denatured proteins were then resolved in the second dimension on 13% polyacrylamide, 0.1%SDS and 5.5 M urea gels (Schagger and von Jagow, 1991; Williams et al., 2003).

Polyclonal antibody raised against human SCO2

A Sco2-specific antibody was generated by injecting rabbits with a synthetic peptide specific for the C-terminal part of human Sco2 (CGRSRSAEQISDSVRRHMAAF). Testing of the specificity of the Sco2 antiserum revealed that affinity purification was not required, and crude serum was used in all subsequent experiments.

Immunoblot analysis

Proteins were electroblotted from the gels on to ImmobilonTM-P PVDF membranes (Millipore) using semi-dry transfer for 2 h at a constant current of 0.8 mA/cm2. Membranes were air-dried overnight, rinsed twice with 100% (v/v) methanol and blocked in PBS and 10%(w/v) non-fat dried milk for 1 h. Primary detection was performed with mouse monoclonal antibodies raised against COX subunits COX1 (A-6403; 1 *μ*g/ml), COX2 (A-6404; 1 *μ*g/ ml), COX4 (A-21348; 2 *μ*g/ml), COX5A (A-21363; 2 *μ*g/ml) and COX6B (A-21366; 1 *μ*g/ml) (Molecular Probes), with rabbit polyclonal antiserum raised against human SCO2 (1:1000) and with monoclonal antibodies raised against the flavoprotein subunit of SDH (succinate:ubiquinone oxidoreductase) (A-11142; 0.1 *μ*g/ml) (Molecular Probes) and the VDAC(voltage-dependent anion channel) (31HL Ab-1; 1.4 *μ*g/ml) (Calbiochem) at indicated dilutions. Blots were incubated with primary antibodies in PBS, 0.3% (v/v) Tween 20 and 1% non-fat dried milk for 2 h. Secondary detection was carried out with goat anti-mouse IgG– horseradish peroxidase conjugate (A8924; 1:1000) (Sigma– Aldrich) or with goat anti-rabbit IgG–horseradish peroxidase conjugate (A0545; 1:2000) (Sigma–Aldrich) in PBS, 0.1% Tween 20 and 1% non-fat dried milk for 1 h. The blots were developed with West Pico Chemiluminescent substrate (Pierce) and exposed to

Kodak BioMax Light films (Kodak). The films were subsequently scanned and digital images were analyzed using the Quantity One application (Bio-Rad Laboratories).

Structural and histological studies – methods[**]

Material

The formalin-fixed and frozen tissues were collected from autopsies carried out in several pathology departments. In rare cases, biopsy samples were available. The samples were embedded in paraffin for histology. For electron microscopy the samples were embedded in Epon – Araldite mixture – and examined using a Jeol 100B microscope. Frozen samples were preferred for biochemistry.

Histochemistry

Cryostat sections 10 μm (for COX histochemistry) or 5 μm (for histochemistry of other respiratory chain enzymes) prepared from frozen tissues were stained for COX, succinate dehydrogenase (SDH) and NADH-tetrazolium reductase (NADH-TR) activity using standard laboratory methods (Lojda et al., 1979).

Immunohistochemistry

Mouse IgG1 anti-prohibitin antibody (LabVision, Fremont, CA USA) and mouse IgG1 anti-mitochondrial antigen antibody (MU213-UC, clone 113-1, Biogenex, San Ramon, CA, USA) working well in paraffin-embedded sections after antigen retrieval (10 mM citrate buffer, pH 7.6, for 3x5 min in a microwave 750 W) were used for staining mitochondria. Primary antibodies were incubated overnight at 4°C, diluted 1:500 and 1:100, respectively. They were detected using a DAKO EnVisionπTM Peroxidase Mouse kit (DAKO, Glostrup, Denmark) with 3,3´-diaminobenzidine as substrate.

[**] these analyses were performed in collaboration with H. Hůlková at Institute of Inherited Metabolic Disorders, 1st Faculty of medicine, Charles University and General University Hospital, Praha

RESULTS AND DISCUSSION

Ad 3) to search selected genes for mutations in the group of our patients with isolated COX deficiency

SCO2 gene

35 patients were screened. Pathological mutations were found in 8 of them. Six children were homozygous and their parents were heterozygous for the missense mutation g.1541G>A that exchanges glutamate for lysine at the position 140 of the amino acid chain (E140K). The patient seven is compound heterozygous for mutations g.1541G>A, and g.1280C>T which results in a stop codon replacing glutamine at position 53 (Q53X). This patient inherited the g.1541G>A mutation from his mother; no DNA was available from his father. In addition, the first five patients are also homozygous, while patient seven is heterozygous, in the S*CO2* gene for the polymorphism g.1182G>C and the silent mutation g.1756A>C. Last found patient is heterozygous for the g.1541G>A and a novel deletion 1518delA causing the frame shift. The protein is then affected at the 132nd amino acid by one base frameshift resulting in the disappearance of the most important Sco2-protein part, the Cu-binding motive at position 133-137. First six cases were published by Vesela et al. 2004. Last two cases are not published yet.

SCO1 gene

Screening of 14 candidates revealed one patient with homozygous mutation c.394G>A in 3rd exon of *SCO1* gene. This mutation changes glycine at the position 132 to serine. Parental samples are not available. This mutation was not present in 200 healthy controls (Vesela, Stiburek et al. 2008, manuscript in preparation).

COX10 and COX15

Among 24 patients for screening of *COX10*, and 22 patients for screening of *COX15* we did not find any harbouring pathological mutation. In *COX10* we found 3 novel nucleotide exchanges (Tab. 5)

Tab. 5 Found novel polymorphisms in *COX10* gene

nucleotide exchange	amino acid exchange	number of patients		% of healthy controls	
		heterozygous	homozygous	heterozygous	homozygous
c.184A>T	T64S	3	0	17	0
c.476G>A	R159Q	10	7	61	18
c.1291C>T	R131W	1	0	9	0

Ad 4) to study the pathology of found mutations

SCO1 gene

In one case, it was found guanine > adenine nucleotide transition at the position 394 of the *SCO1* cDNA.

The girl was born at the 39th week of gestation with a birth weight of 2200 g and length 46 cm. She was classified as a hypotrophic newborn with a tendency to hypoglycemia. Progressive hypotony and psychomotor retardation were developing since early postnatal period. Echocardiography at the age of 5 months revealed progressive hypertrophy of left ventricle and a sonography revealed atrophy of central nervous system. Liver enlargement and biochemical investigation at the age of 6 month indicated hepatopathy. She died due to cardiac failure at the age of 6 months. In comparison with the only published patients (Valnot et al., 2000a), the clinical symptoms are relatively similar except the progressive cardiological involvement. The published patient has no evidence of cardiological involvement except episodes of bradycardy. The reason for differences between the described case and our patient may be in shorter live span of 2 months, while the hypertrophy at our patient was recognized at the age of 5 months. The liver enlargement was not observed at our patient till the age of 6 month; the published patient showed this symptom four months earlier.

Dominant laboratory finding was severe lactic acidemia (fasting lactate 3.61 mmol/l, normal value <2.3 mmol/l) with even higher postprandial level (7.72 mmol/l). The urinary organic acid profile showed elevated excretion of 2-oxoglutarate (1150 mg/g creatinine, normal value <200 mg/g creatinine), lactic acid (103 mg/g creatinine, normal value <60 mg/g creatinine) and other Krebs cycle intermediates (fumaric acid 185-193 mg/g creatinine, normal values <15 mg/g creatinine, malate 75 mg/g creatinine, normal value <15 mg/g creatinine). Mild ketonuria (3-hydroxybutyric acid 107 mg/g creatinine, normal value <100 mg/g creatinine) and dicarboxylic aciduria

(due to MCT oil administration) was also found. These findings including the elevated Krebs cycle intermediates correspond to the published data (Valnot et al., 2000a).

The activity of COX was markedly low in isolated muscle mitochondria obtained from a muscle biopsy at the age of 6 months (11,42 nmol/min/mg protein, reference range of age related controls 25-120 nmol/min/mg protein). The activity of citrate synthase, the control enzyme, was normal.

The mutation changes glycine at the position 132 of the amino acid chain to serine. According to the multispecies alignment (Fig.2), it is clear that guanine as well as glycine at that position is highly conserved. 200 healthy controls were examined and the mutation was not present at any allele. The pathology of this mutation can be explained due to its localization: region among leucine 108 and isoleucine 131 is described as an important for dimerization of functional Sco1 protein (Leary et al., 2004). Replacement of nonpolar glycine at the closely contiguous position 132 by serine with the uncharged but polar side chain may have influence on the stability of the dimer itself.

Fig.2 Genomic sequence alignment (Ensembl) of the specific region

Source: Homo sapiens chromosome:NCBI36:17 part of 3rd exon of *SCO1* gene; Bos taurus chromosome:Btau_3.1:19; Gallus gallus chromosome:WASHUC2:18; Macaca mulatta chromosome:MMUL_1:16; Monodelphis domestica chromosome:BROADO5:2; Pan troglodytes chromosome:CHIMP2.1:17; Mus musculus chromosome:NCBIM37:11; Rattus norvegicus chromosome:RGSC3.4:10; Drosophila melanogaster CG8885.2L; Caenorhabditis elegans C01F1.2 II; Saccharomyces cerevisiae YBR037C II

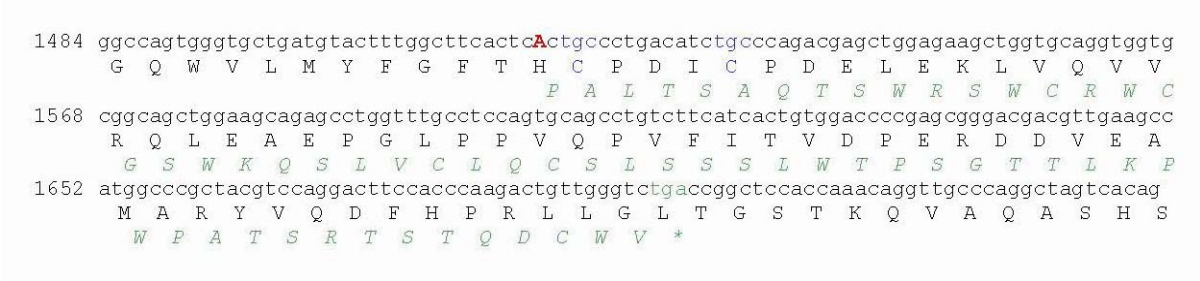
SCO2 gene

Genotype - phenotype correlation

The mutation g.1280C>T was described previously in literature (Papadopoulou et al., 1999). This transition is creating a premature stop codon at level of glutamine 53 (Q53X).

A novel one base deletion at the position 1518 leads to the frame shift. The protein is affected from the 132nd amino acid, and it results in vanishing of the most important part of the Sco2 protein, the Cu-binding motive CxxxC at position 133-137. If the affected sequence would be translated, the newly synthesized protein would miss the Cu-binding motive, which seems to be crucial for its function, and would be shorter due to the hypothetical premature stop codon (Fig.3).

Fig 3. The possible effect of deletion g.1518delA.



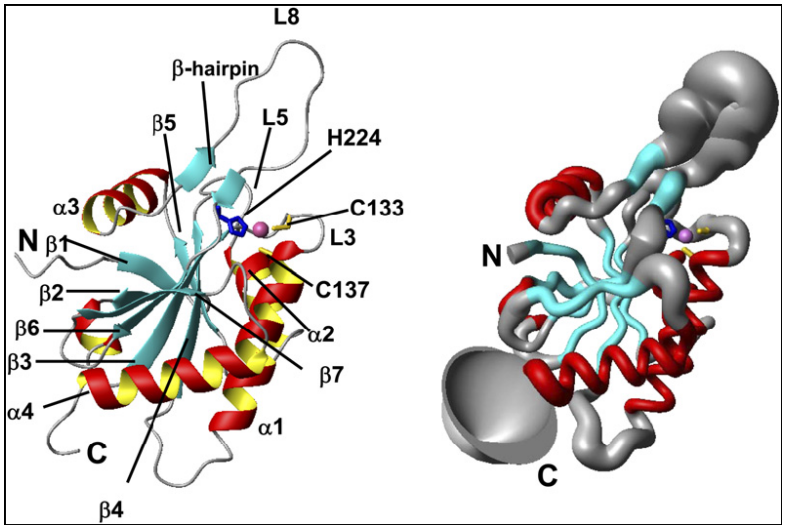
1st row – part of the cDNA sequence of *SCO2* gene (affected base in red; Cu-binding motif in blue)
2nd row appropriate translation
3rd row hypothetical translation after the deletion of the 1518 base (in green)

Transition g.1541G>A cause replacement of glutamic acid by lysine at the position 140 of the amino acid chain. Banci et al. 2007 (Banci et al., 2007a) explains its pathogenity based on structural characterization of the Sco2 protein: “Glu140, located in helix α1 and essentially not solvent exposed, is involved in a salt bridge with Lys143, which is disrupted in the Glu140Lys mutant. In addition, the introduction of a longer side chain in a buried region could locally destabilize side-chain packing between helix α1 and the facing β sheet. Because Glu140 is relatively close to the copper binding Cys137, the possible structural rearrangements induced by the mutation could affect both the copper binding properties and protein stability, factors which both influence protein function, thus possibly rationalizing at the molecular level the mutant misfunction.” (Banci et al., 2007a). For better illustration see Fig.5.

Fig.5 Structure of human Cu(I) Sco2 (Banci et al., 2007a)

On the left side, the average structure of the lowest-energy ensemble is shown. The metal binding residues Cys133 and Cys137 are shown in yellow, and His224 is shown in blue. The copper(I) ion is depicted as a pink sphere.

On the right, the superimposition of 30 lower-energy structures of Cu(I)HSco2 is represented as a tube whose radius is proportional to backbone rmsd. α helices and β strands are colored in red and cyan, respectively.



The clinical data of our patients with mutations in the *SCO2* gene are summarized in Tab.6.

Tab. 6 Clinical data in eight children with isolated COX deficiency and mutations in the *SCO2* gene

Patient		Onset of symptoms	Inspiratory stridor	Infantile encephalopathy	Hypotonia	HCMP*	Brain atrophy	Respiratory** insufficiency	Age at death
group A	1.	3 mo	++	++	++	-	microcephaly	last 2 mo	8 mo
	2.	6 wk	-	++	++	-/+	?	last 2 mo	12 mo
	3.	4 mo	++	++	++	-	++	last mo	15 mo
	4.	6 mo	+	++	+	-	++	last mo	8 mo
	5.	5 mo	-	++	hypertony	-	++	last mo	9 mo
	6.	3 mo	-	++	++	-/+	++	since 6th mo	alive***
group B	7.	birth	-	++	+	++	++	since birth	7 wk
	8.	birth?	++	?	++	++	++	since birth?	3 mo

* HCMP: hypertrophic cardiomyopathy.

** artificial ventilation in an intensive care unit.

*** last information at the age of 7 mo

SCO2 mutations are commonly associated with fatal infantile hypertrophic cardio-encephalo-myopathy. Looking at the clinical data of our patients (tab 6), who represent the biggest group of patients with mutations in *SCO2* gene published so

far, it brought us to deeper study of ours and literature findings. We found out, that the patients with *SCO2* mutations can be divided into two subgroups due to their phenotype, which surprisingly very well corresponds with their genotypes.

First group (group A) represents patients harbouring the homozygous state of the prevalent mutation E140K (g.1541G>A). Group A include six of our patients (Patient 1 to 6; P1 – P6) and three published cases (Jaksch et al., 2001a).

The second group (group B) includes patients with heterozygous form of E140K combined with another mutation. The second group is composed of patient 7 (P7; g.1280C>T), patient 8 (P8; g.1518delA) and all other published cases (Jaksch et al., 2000; Leary et al., 2006; Papadopoulou et al., 1999; Sacconi et al., 2003; Salviati et al., 2002b; Sue et al., 2000; Tarnopolsky et al., 2004; Tay et al., 2004)

Various polymorphism and silent mutations were described in the *SCO2* gene (Jaksch et al., 2000; Jaksch et al., 2001b; Papadopoulou et al., 1999). Our patients from group A were all homozygous, and patients from group B heterozygous for the polymorphism g.1182G>C and the silent mutation g.1756A>C in the *SCO2* gene.

The first clinical symptoms of the disease in group A occurred early in the neonatal period, but compare to the first symptoms manifested in group B, there were delayed.

The common findings in group B are encephalopathy and fatal hypertrophic cardiomyopathy with early onset in the neonatal period leading to death within the first 3 month of life.

In contrast, all children in group A had the early postnatal adaptation uneventful. The first clinical symptoms including progressive infantile encephalopathy with muscle weakness and respiratory distress developed between 3rd and 10th month of age. All of them died between 8 and 18 month of age due to respiratory failure. No hypertrophic cardiomyopathy was present in our patients, and the onset of cardiomyopathy in patients described in the literature was delayed after the age of 8–18 months (Jaksch et al., 2001a).

Two patients in literature (Salviati et al., 2002b; Tarnopolsky et al., 2004) and our patients P6 and P8 were also classified as Werdnig-Hoffmann disease - like phenotype, but all patients were unsuccessfully investigated for mutation in the *SMAI* gene before the diagnosis of COX deficiency was acknowledged.

On neuroimaging using CT and/or MRI, severe cortical atrophy was found in all children from both groups, but the typical necrotic lesions in the basal ganglia associated with Leigh syndrome were never observed.

Among the common laboratory findings belong increased levels of lactate and alanine in both blood and cerebrospinal fluid. COX activities were decreased in the skeletal muscle tissues, but the activities measured in cultivated fibroblasts were about normal. That is in contrast to the patients with Leigh syndrome due to mutations in the *SURF1* gene, where COX deficiency is observed also in cultivated fibroblasts.

Our observations were published in original paper Vesela et al. 2004. The patient (P6) was diagnosed in April 2008 and the data are collected to be published soon (Klement et al., 2008, manuscript in preparation).

Although the clinical course of the disease may depend on the type of mutation in the *SCO2* gene, the prognosis is unfavorable in all affected children. Functional consequences of *SCO2* mutations are still poorly understood, but they are related to changes in the amount and composition of COX molecules in different cells.

There were available a few frozen samples of different types of tissue from our patients with isolated COX deficiency due to *SCO2* mutations. It opened new possibilities of further studying of pathological consequences in different tissues on biochemical and histochemical levels, which were described in original articles and presented at the international conferences (Stiburek et al., 2005).

Biochemical studies

Samples harbouring mutations in *SCO2* were available from P2, P5 – both group A (homozygous for g.1541G>A) - and P7 representing group B (g.1280C>T / g.1541G>A). While there was not enough material of different tissues to perform all the measurements in each of both *SCO2* groups and then to obtain relevant comparable results, we decided to moderate the aim and compare the findings to the results obtained from samples with COX deficiency due to mutations in *SURF1* gene as well.

There were chosen relevant samples of three patients with *SURF1* mutations. For better orientation, we marked them as patient 9-11. Patient 9 (P9) was a compound heterozygote for the combined frame shift deletion-insertion mutation in

SURF1 c.312_321del 311_ 312insAT, resulting in the formation of a premature termination codon, and a c.821_839del18 deletion in *SURF1* leading to highly unstable mRNA (Williams et al., 2001a). Patient 10 (P10) was homozygous for the frame shift deletion c.845_846delCT leading to the formation of a premature termination codon, and patient 11 (P11) was homozygous for a c.688C>T nonsense substitution in *SURF1* leading to the formation of a premature termination codon truncating the protein at Arg230 (Pecina et al., 2003).

Activities of COX in SCO2 and SURF1 patient tissues

To determine the residual COX activity of the mitochondrial preparations, we expressed COX activity relative to the activity of the mitochondrial marker enzyme, citrate synthase (CS). COX/CS ratio presents values of COX activity normalized to CS activity as a percentage of the mean reference range. Severe isolated defects of COX activity were found in the *SCO2* patient heart (8-34% COX/CS), skeletal muscle (19-28%) and brain (13-18%), whereas in cultivated fibroblasts (62-100%) and liver (64-100%) the activity was in reference range or border low. There were no significant differences between the *SCO2* groups A and B. In contrast, severe reduction of COX activity was found in all of our *SURF1* fibroblast cultures (6-14% COX/CS) (Tab.7).

Tab.7 COX activity in various tissues of SCO2 patients P2, P5, P7 and SURF1 patients P9–P11

	P2	P5	P7	P9	P10	P11
Tissue	COX/CS activity (%)					
Heart	8	34	30	n.d.	n.d.	n.d.
Muscle	19	28	21	11	22	9
Brain	16	18	13	20	n.d.	n.d.
Liver	76	64	100	n.d.	n.d.	n.d.
Fibroblasts	62	82	100	12	6	14

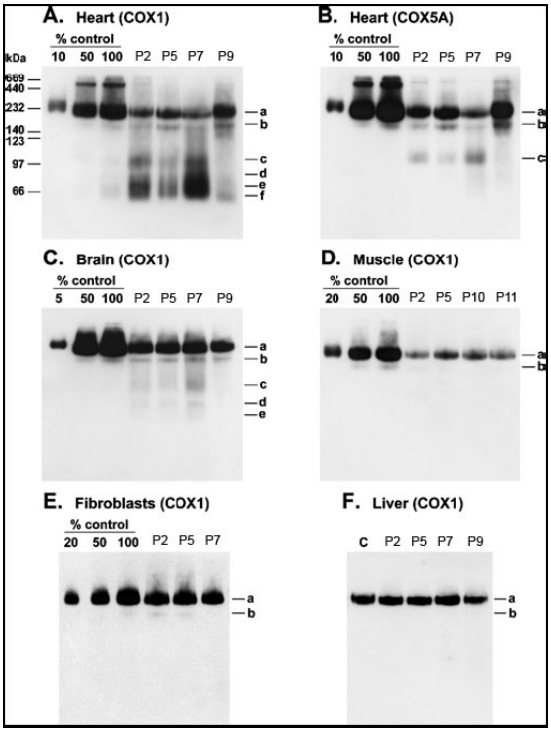
Values of COX activity normalized to CS activity are expressed as a percentage of the mean reference range (COX/CS activity). n.d., not determined.

Steady-state levels of COX holoenzyme in SCO2 and SURF1 patient tissues

All mitochondrial preparations used in the present study were balanced on the basis of the immunoblot signal of the mitochondrial inner membrane protein complex SDH. To determine the residual steady-state levels of COX holoenzyme in tissues of patients as a percentage of control values, dilutions of control mitochondria were

loaded on the same gels. Mitochondrial samples were resolved using BN-PAGE and subsequently probed with an anti-COX1 antibody. In heart mitochondria from patients P2, P5, P7 and P9, the steady-state levels of COX holoenzyme were found to be approx. 25, 30, 10 and 40% of control values respectively (Fig.6A and 6B). Mitochondria from basal ganglia of patients P2, P5, P7 and P9 contained approx. 20 and 15% of residual holoenzyme respectively (Fig.6C). In skeletal muscle from patients P2, P5, P10 and P11, the holoenzyme levels were approx. 10, 20, 15 and 10% of control values respectively (Fig.6D). In primary fibroblasts, the steady-state levels of COX holoenzyme were found to be approx. 70% of control values in the case of patients P2 and P5, approx. 60% in the case of patient P7 (Fig.6E) and approx. 15% in the case of patients P10 and P11. The liver samples of *SCO2* patients (P2, P5, P7) were the least affected and contained similar steady-state levels of COX holoenzyme to control samples, whereas in *SURF1* patient liver (P9) the holoenzyme was found to be approx. 80% of control values (Fig.6F). The only slight difference between group A and B might be seen in the steady-state levels of COX holoenzyme in the sample of heart mitochondria, where there is more profound decrease in the patient from group B.

Fig.6 Steady-state levels of COX holoenzyme and subcomplexes in tissues and fibroblast cultures of SCO2 and SURF1 patients.



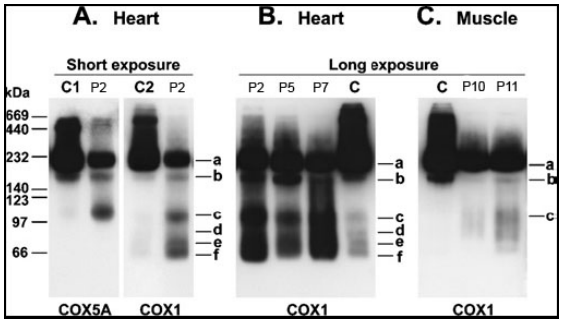
Mitochondrial fractions from SCO2 (P2, P5, P7) and SURF1 (P9–P11) patient samples were resolved by BN-PAGE (8–15% polyacrylamide), electroblotted on to PVDF membranes and probed with monoclonal antibodies specific for subunits COX1 (**A**, **C**–**F**) or COX5A (**B**). The amount of protein loaded per lane (~5 μg) was normalized to SDH. Three aliquots of control mitochondria corresponding to indicated dilutions of control samples were loaded on the same gels. Immunoreactive material was visualized by chemiluminescence. The positions of COX holoenzyme (a), COX subcomplexes (b–f) and molecular-mass standards (kDa) are indicated.

Subcomplexes of COX in SCO2 and SURF1 patient tissues

Mitochondrial preparations from various *SCO2* and *SURF1* patient tissues, primary fibroblast cultures and control samples were resolved using either BN-PAGE or two-dimensional BN/SDS/PAGE and subsequently probed with anti-COX subunit-specific monoclonal antibodies in order to detect the presence and possible accumulation of COX subcomplexes and to uncover their subunit composition. In addition to holoenzyme complex (Fig.6A and 8A, complex a), heart samples of *SCO2* patients contained eight distinct COX subcomplexes (b–i). Prolonged exposure of the blots revealed the presence of six of them (b–f and i) also in control heart samples (Fig.7B and 8A). Subcomplexes c–i were found in *SCO2* heart samples at highly accumulated levels, whereas only subcomplexes b and f were found slightly increased in the heart of the *SURF1* patient (Fig.6A). Mitochondria from *SCO2* skeletal muscle contained increased levels of seven distinct subcomplexes (Fig.8C, c–i), apparently identical with that found in *SCO2* patient heart. Their steady-state levels were, however, found to be substantially lower in this tissue (Fig.6D), and their full detection thus required higher protein loads (~50 *μ*g) and longer exposure times (Fig.8C). In line with this, control heart samples contained substantially higher steady-state levels of COX subcomplexes than skeletal muscle controls (Fig.7B and 7C). Also, *SCO2* brain samples revealed accumulated COX subcomplexes of approx. 10–120 kDa, detectable mainly with anti-COX1 and anti-COX5A antibodies, but their profile differed from that found in *SCO2* heart and skeletal muscle mitochondria (Fig.8A-C). In particular, the COX4 signal in the brain was very weak in this region. In contrast with *SCO2* patient samples, we did not detect any accumulated subcomplexes in *SURF1* patient brain (Fig.6C). In skeletal muscle of *SURF1* patients, we found substantially decreased levels of subcomplex b and faint accumulation of subcomplexes with similar electrophoretic mobility to subcomplexes c–f from *SCO2* samples (Fig.7C). Mitochondria from *SCO2* patient kidney contained moderately increased subcomplexes with migration similar to subcomplexes c–f and i from *SCO2* patient heart (Fig.8D). We did not detect any conclusive accumulation of COX subcomplexes in *SCO2* patient fibroblasts (Fig.6E and 8E). Alignment of parallel run immunoblots probed with different antibodies indicated, together with immunoblots of two-dimensional native/denaturing gels, that subcomplex b consists of at least COX1, COX2, COX4, COX5A and COX6B. Subcomplex c comprised at

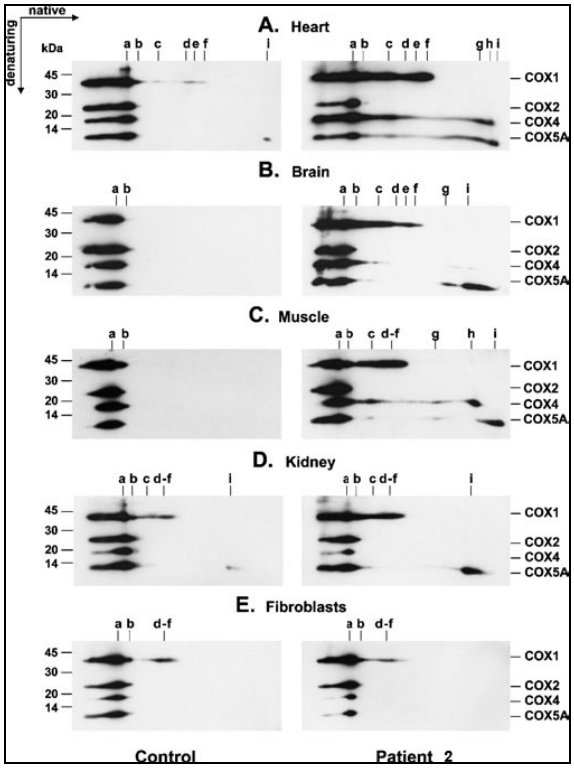
least subunits COX1, COX4 and COX5A, whereas subcomplexes d–f were recognized solely with an anti-COX1 antibody (Fig.6-8). Subcomplex g was detectable with both anti-COX4 and anti-COX5A antibodies, whereas subcomplexes h and i were recognized only with single anti- COX4 and anti-COX5A antibodies respectively (Fig.8A-C). Low-molecular-mass subcomplexes g–i were not detectable on immunoblots of one-dimensional native gels, since the polyacrylamide gradient used (8–15%) was optimal for fractionation of subcomplexes b–f, while lower-molecular-mass polypeptides were allowed to migrate out of the gel.

Fig.7 COX subcomplexes in control heart, heart of SCO2 patients and skeletal muscle of SURF1 patients



Mitochondrial fractions from SCO2 patient heart (P2, P5 and P7) (**A**, **B**), SURF1 patient skeletal muscle (P10 and P11) (**C**) and control samples were resolved by BN-PAGE (8–15% polyacrylamide), electroblotted on to PVDF membranes and probed with monoclonal antibodies specific for subunits COX1 or COX5A. The amount of protein loaded per lane (~5 *μ*g) was normalized to SDH. Immunoreactive material was visualized by chemiluminescence. The positions of COX holoenzyme (a) and COX subcomplexes (b–f) and the molecular-mass standards (kDa) are indicated.

Fig.8 Subunit composition of COX subcomplexes in various tissues of the *SCO2* patient P2



Mitochondrial fractions (10–50 *μ*g) from various tissues of SCO2 patient P2 and control samples were resolved using two-dimensional BN/SDS/PAGE, electroblotted on to PVDF membranes and probed simultaneously with monoclonal antibodies specific for subunits COX1, COX2, COX4 and COX5A. Sample loads and exposures of films to the blots were chosen such that the signals corresponding to holoenzyme complex *a* were of similar intensitieswithin both kontrol and patient immunoblots. Immunoreactive material was visualized by chemiluminescence. The polyacrylamide gradient used in the first dimension (BN) was 8–16% for (**A**, **B**) and 10–18% for (**C**–**E**). The positions of COX holoenzyme (a) and COX subcomplexes (b–i) and the migration of molecular-mass standards (kDa) are indicated.

Steady-state levels of SCO2 protein in various tissues of SCO2 patients

Mitochondrial fractions and fibroblast lysates were resolved using SDS/PAGE and subsequently probed with polyclonal antiserum raised against human SCO2. Equal loading was verified with an antibody raised against the mitochondrial outer membrane protein VDAC. The SCO2 protein was undetectable in all *SCO2* brain samples and in heart of patients P5 and P7. In heart of patient P2 and liver of patients P2 and P7, the levels of mutant Sco2 were approx. 5% of control values. In fibroblasts of patients P2, P5, P7 the residual Sco2 was approx. 20% of control values, while in liver of patient P5 it was approx. 10% of control values.

Although both Sco2 and Surf1 proteins are thought to act at a similar stage of COX assembly, patients carrying mutations in respective genes present with distinct clinical phenotypes. Quantitative immunoblot analysis of native gels revealed tissue-specific COX assembly defects in all patients studied that corresponded to the enzyme activity measurements. The steady-state levels of mutant Sco2 protein were found severely reduced in all the probed *SCO2* patient tissues. The subunit composition of COX subcomplexes identified demonstrates the involvement of human SCO2 protein in biogenesis or maintenance of COX2.

The COX holoenzyme was repeatedly shown to be reduced to approx. 15% in *SURF1* patient fibroblasts. Although skeletal-muscle samples of our *SURF1* patients revealed a similar decrease in COX holoenzyme to cultured fibroblasts, SURF1-deficient heart and liver contained substantially higher levels of residual holoenzyme. In contrast, the tissue-specific consequences of *SCO2* mutations, mainly the profound difference between the residual COX activity in skeletal muscle and fibroblasts.

We show that despite very low levels of mutant protein the livers of *SCO2* patients display practically no reduction of fully assembled COX, corresponding to high residual activity.

The precise molecular basis of tissue-specific consequences of *SCO2* and *SURF1* mutations remains unresolved. In addition to different levels of COX holoenzyme, variable levels of subcomplexes were found among different tissues, although some of them displayed the same residual level of the holoenzyme (e.g. heart and muscle). This is likely to be attributable to different rates of clearance of partially assembled or unassembled subunits. The tissue-specific pattern of

assembly defects only partially overlaps with the expression of particular tissue-specific isoforms of COX subunits, suggesting the involvement of a rather different mechanism. In our patients, the mutant Sco2 protein was almost undetectable in brain and heart with profound COX deficiency, whereas the liver, and particularly fibroblasts, contained small but significant amounts of residual Sco2. However, we find it unlikely that this minor difference could account for the distinct biochemical and clinical involvement of these tissues, unless there is a pronounced difference in 'spare capacity' of Sco2 for copper delivery to the Cu_A centre in these tissues. Therefore we speculate that tissue-specific consequences of *SCO2* and *SURF1* mutations, in terms of both holoenzyme and subcomplex levels, suggest the existence of tissue-specific functional differences of these proteins that may have evolved to meet different requirements for the regulation of COX biogenesis.

Structural and histochemical studies

Patient samples for these studies were divided into two groups due to the mutation type in *SCO2* gene. Group A represents tissues of patients harbouring the homozygous state of the prevalent mutation E140K (g.1541G>A). Group B collects samples with heterozygous form of E140K combined with other mutation (g.1280C>T or g.1518delA) (Vesela et al., 2008).

Some of them were available for histochemical analysis (Tab. 8).

Tab. 8 List of tissues available for analysis

	Group A phenotype					Group B phenotype	
	Siblings			Siblings			
	Patient 2 male 12 mo death	Patient3 female 15 mo death	Patient 5 female 9 mo death	Patient 1 male 8 mo death	Patient 1b male not known	Patient 7 male 7 wk death	Patient 8 female 11wk death
CNSa	P/F BC+HC	P	P/F BC+HC	P	0	P/F BC	P
Regions of CNS	C, Bg, CRBL, brain stem, MS	CRBL, MS	C, Bg, CRBL*, brain stem	C, Th, Bg, CRBL, brain stem		C, MS	Bg
Peripheral nerve[b]	0	0	0	0	EM skin nerve	0	EM sural nerve
Skeletal muscle [a/b]	F BC+HC	F+EM BC+HC	F BC+HC	P	F BC	P/F BC	P/F+EM BC+HC

Heart[a]	P/F BC+HC	0	P/F BC+HC	P	0	P/F + EM BC	P
Liver [a/b]	P/F+EM BC+HC	0	P/F BC+HC	P	0	P/F BC	F BC+HC
Other tissues	Kidney P/F (HC)	0	Kidney P/F (HC)	0	0	Retina P	0

Abbreviations: CNS, central nervous system; C brain cortex; Th, thalamus; Bg, basal ganglia; CRBL, cerebellum; MS, spinal medulla; F, frozen tissues; P, formalin-fixed paraffin-embedded tissues; EM, biopsy for electron microscopy; BC, COX deficiency proven biochemically; HC, COX deficiency confirmed histochemically. 0 tissues not available.
*Dentate nucleus was available only in this case.
Patient 1b is a brother of patient 1, who were not investigated in any other studies only in structural and histochemical.

Central and the peripheral nervous systems

In both groups there were regressive changes characterized by shrinkage of neurons and neuronal depopulation accompanied by gliosis and variable neocapillarization. The changes were expressed with different intensity in the areas examined (for sample availability in individual cases see Tab.8). Maximal neuronal depletion was focally expressed in the cerebellar cortex affecting both granular and Purkinje cell layers, associated with marked Bergman astrogliosis. Severe neuronal degeneration and astrogliosis were also seen in the dentate nucleus (available in one case only). High degree neocapillarization accompanied by loss of individual cells was present in basal ganglia in both groups. Necrosis of the type seen in Leigh syndrome (Powers and DeVivo, 2002) was not observed. In other parts of the brain (cortex, thalamus and brain stem), there was only patchy loss of neurons. The spinal medulla motoneurons were moderately affected in both groups. The number of mitochondria was not generally increased, with the exception of large striatal neurons and some spinal motoneurons, the perikarya of which harboured numerous fine eosinophilic granules strongly stained with both antimitochondrial antibodies. COX activity was also uniformly decreased in both samples available for histochemistry.

Neuronal involvement is non-specific in terms of morphology. It is widespread throughout the central nervous system and corresponds to the category of polioencephalopathy described in mitochondrial disorders (Powers and DeVivo, 2002). Structural changes in the nervous system were developed in both groups, in spite of shorter average survival of patients in group B.

The sample of retina was available in one patient from B group. There was pronounced shrinkage and loss of neurons restricted to the ganglion cell layer, whereas the number of neurons in both outer and inner nuclear layers was not significantly altered. There were large eosinophilic granular aggregates strongly stained for mitochondria in the retinal photoreceptors. They were concentrated

between the photoreceptor inner and outer segments. Discrete aggregates were seen in neurits in the external plexiform layer.

Ultrastructural analysis of *peripheral nerves*, realized in two patients (one of each phenotype group, Tab.8), showed axonal degeneration and demyelinization. The sural nerve from the compound heterozygous patient also showed signs of remyelinization and axonal sprouting in spite of the shorter course of disease in this case. No mitochondrial abnormalities were observed.

Here we show for the first time that affection of retinal neurons may be an integral part of the neuropathology in COX deficiency due to *SCO2* mutations. The ultrastructure of the mitochondrial aggregates in the zone of photoreceptors could not be evaluated for technical reasons. To the best of our knowledge there is no evidence of similar mitochondrial alteration in mitochondrial retinopathies studies (McKechnie et al., 1985). Alterations of peripheral nerves suggest the presence of peripheral neuropathy also involving sensitive fibers (sural nerve). Our pathological findings (sensitive neuropathy, retinopathy) exceed the range of clinical symptomatology.

Skeletal muscle and the skeletal muscle involvement

The spectrum of findings in tissues from the patients of group A ranged from relatively mild structural changes (atrophy of individual fibres, both angulated and rounded, occasional fibre hypertrophy and rare muscle fibre destruction) to fully developed neurogenic atrophy. The histochemistry showed irregular decrease in COX activity in samples of three patients (Tab.8). In all of them, both SDH and NADH-TR were highly active, without having a regular normal checkerboard pattern. Groups of atrophic angulated fibres displayed typical enhanced activities of both dehydrogenases. In one case in group B (death at 11 weeks), signs of neurogenic atrophy were combined with myopathic changes (marked hypertrophy of muscle fibres in comparison to age-matched samples, frequent internalized nuclei, variation in fibre size). COX activity was not detectable histochemically. In the second case (death at 7 weeks), the pathology was limited to increased variation in fibre size with occasional enlarged rounded fibres. Ragged red fibres and ultrastructural mitochondrial abnormalities were absent in both groups.

With regard to spinal motoneuron degeneration and COX deficiency in muscle fibres proven histochemically and biochemically, we explain the skeletal muscle

pathology as a consequence of ‘‘double hit’’ – neurogenic lesion and metabolic affection. The range of structural alterations in our patients may be caused by differences in skeletal muscle sampling, which may not match lesions of the spinal motoneurons. Isolated decrease in COX activity secondary to neurogenic muscle atrophy is not likely, as it is associated with decrease in the activity of other respiratory chain enzymes, including SDH (Berger et al., 2003).

Heart and the cardiac involvement

The histology showed no signs of cardiac hypertrophy in group A and only moderate multiplication of mitochondria without increase in their size, demonstrable by specific staining. COX activity tested histochemically was found to be uniformly decreased (samples from two cases available). In group B there was marked cardiac concentric hypertrophy of both ventricles with three and four-fold increase in weight against age-matched controls. The histology showed hypertrophy of cardiocytes with enlarged hyperchromatic and often doubled nuclei. Mitochondria were increased in number and many of them were significantly enlarged. Ultrastructurally they were frequently aggregated densely with very close contacts of their external membranes. In smaller mitochondria, the cristae were short and condensed. In larger mitochondria (4–5 mm in diameter) the cristae were loosened, longer and unevenly oriented. Some of them were tubular; others were narrow, densified, linear and rigid. Single or several lucent intramitochondrial vacuoles (lipid? glycogen?) with marked tendency to fusion were sometimes seen. Crystalloid formations were absent. Profound COX deficiency could be confirmed biochemically in one case (patient 7).

The cardiac involvement due to hypertrophic cardiomyopathy was linked to compound heterozygosity for E140K and a nonsense mutation, and was responsible for the severe, rapidly progressive clinical course. No significant cardiac involvement was apparent in our patients homozygous for E140K.

Kidney

Samples were available from two patients of group A. The histology was normal, without any significant increase in the number of mitochondria. Strong histochemical activity of COX was comparable with controls.

Liver

There was neither hepatomegaly nor increase in the number of mitochondria in the histology of available samples from patients of group A. In two cases there was microvesicular steatosis. In one of them it was associated with increase in the amount of glycogen. In the third case the histology was normal. Liver biopsy available in one case showed no abnormalities of the mitochondrial ultrastructure. In group B there was centrolobular venostasis associated with necrosis of individual hepatocytes and discrete intracellular cholestasis (patient 7). Microvesicular steatosis was seen in patient 8. In two samples (patients 2 and 8) there was a questionable decrease in histochemically detectable COX activity, corresponding to the biochemical results.

So no obvious disease-specific structural alterations were detectable with the exception of borderline decrease in COX activity in some cases. Alteration of the liver parenchyma in group B is most probably secondary to venostasis due to prolong heart failure before death of the investigated patients. These findings together with the absence of any pathology in the kidney correspond well with the clinical and biochemical data. Although Sco2 protein is expressed in all tissues ubiquitously (Horng et al., 2005), the phenotype resulting from *SCO2* mutations shows decreased COX activity and affliction in certain tissues only; these mutations cause encephalocardiomyopathy (Jaksch et al., 2000; Leary et al., 2006; Papadopoulou et al., 1999; Sacconi et al., 2003; Salviati et al., 2002a; Sue et al., 2000; Tarnopolsky et al., 2004; Tay et al., 2004). The reason for the tissue sensitivity and specificity is not clearly understood so far.

In terms of pathology the structural phenotype of COX deficiency due to mutations in *SCO2* in the studied set of patients can be characterized as neuromuscular disorder with two subvariants differing in the degree of cardiac involvement.

CONCLUSIONS

Clinical course of the isolated cytochrome c oxidase deficiency is very heterogenous. The results of our studies enabled:

- better understanding of biochemical and functional impact of nuclearly encoded assembling proteins on biogenesis of cytochrome c oxidase
- better understanding of cytochrome c oxidase deficiency manifestation in morphology and function of the cells
- better understanding of natural course of the disease in patients with nuclearly encoded isolated cytochrome c oxidase deficiency
- appropriate genetic counseling and eventual prenatal diagnostics in the affected families

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BN-PAGE	blue native polyacrylamide gel electrophoresis
bp	base pair
BSA	bovine serum albumin
c.	coding
cAMP	cyclic adenosine monophosphate
cDNA	coding DNA
CNS	central nervous system
COX, CcO	cytochrome *c* oxidase
Cox1; COX I; CO I	cytochrome *c* oxidase subunit 1
COX10	cytochrome c oxidase assembling gene 10
COX15	cytochrome c oxidase assembling gene 15
Cox2; COX II; CO II	cytochrome c oxidase subunit 2
Cox3; COX III; CO III	cytochrome c oxidase subunit 3
CS	citrate synthase
CSF	cerebrospinal fluid
CT	computer tomography
Cyt c	cytochrome c
DDM	n-dodecyl □-D-maltoside
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphosphate
EDTA	ethylene diamine tetraacetic acid
FAD^+ and $FADH_2$	flavinadenine dinucleotide (reduced and oxidized forms)
g.	genomic
gDNA	genomic DNA
GDP	guanosine diphosphate
GTP	guanosine triphosphate
LRPPRC	leucine-rich PPR motiv containing protein
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA

NAD^+ and $NADH+H^+$	nicotinamid adenine dinucleotide (reduced and oxidized forms)
NADH-TR	NADH tetrazolium reductase
nDNA	nuclear DNA
OXPHOS	oxidative phosphorylation system
PBS	phosphate buffer saline
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SCO; SCO1; SCO2	syntase of cytochrome c oxidase; protein/gene 1; protein/gene 2
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulphate
SDS/PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SMA I	spinal muscular atrophy type I
SURF1	surfei locus protein 1
TIM	translocase of the outer membrane
TOM	translocase of the inner membrane
UTR	untranslated region

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